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Detection of *p*-Aminobenzoylpoly(γ-glutamates) Using Fluorescamine[1]

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Fluorescamine reacted with the aromatic amines aniline, *p*-aminobenzoic acid, and *p*-aminobenzoylpoly(γ-glutamate) to form adducts which exhibited 50- to 100-fold greater fluorescence than amino acid adducts. This property has been exploited in a simple assay for *p*-aminobenzoylpoly(γ-glutamate) which is sensitive enough to detect as little as 25 pmol/ml without the use of radioactive label. The *p*-aminobenzoylpoly(γ-glutamates) created in the reductive cleavage of crude extracts were located and quantitated in the eluate from DEAE-Sephadex using fluorescamine. Depending on cellular folic acid levels, from 0.1 to 1 g of cell paste or tissue was required for reliable quantitation. Assignments of the polyglutamate chain length were confirmed using amino acid analysis, partial acid hydrolyses, and γ-glutamylcarboxypeptidase treatment. The size and relative amounts of the various *p*-aminobenzoylpoly(γ-glutamates) found in yeast, liver, and various bacteria were consistent with literature data.

A multiplicity of oxidation states and polyglutamate lengths has complicated the analysis of natural folate pools. The microbiological assay, before and after γ-glutamylcarboxypeptidase treatment of ion-exchange chromatography eluates (1,2) has proven to be a tedious, albeit useful, tool and has prompted a search for more convenient analytical procedures. The development of procedures for the cleavage of the folate molecule has somewhat simplified this search since the resulting pABAglu$_n$[3] fraction can be simultaneously separated and sized using ion-exchange resins (3–7). In order to reduce the time and number of manipulations involved in ion-exchange chromatography, Brody *et al.* (8) have described a polyacrylamide gel chromatography procedure which separated and sized the azo dye derivatives of pABAglu$_n$ prepared by the method of Bratton and Marshall (9). More recently Shane (10) has used this same procedure to characterize the pABAglu$_n$ component from *Corynebacterium* sp. Furthermore, this procedure and a slightly modified version (7) had the advantage that the large molar absortivity of the azo dye derivatives made it possible to assay the pABAglu$_n$ pool of rat liver without resorting to radioactive label. As illustrated by Tyerman *et al.* (7) the slow uptake and metabolism of labeled precursors could result in a slightly different apparent composition of polyglutamate lengths.

Fluorescamine has been shown to form fluorescent derivatives with primary amines and has greatly improved the sensitivity of amino acid and protein detection (11,12). This report describes the reaction of pABA and pABAglu$_n$ with fluorescamine to produce a fluorescent adduct which can be detected in nanomolar amounts. Published extraction (8,10,13) and chromatography (3–7) techniques have been adapted with only minor modifications to allow the use of flu-

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[3] Abbreviations used: pABA, *p*-aminobenzoic acid; o-ABA, *o*-aminobenzoic acid; pABAglu$_n$, *p*-aminobenzoylpoly(γ-glutamate).

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TABLE 1

FLUORESENCE VALUES OF VARIOUS FLUORESCAMINE–PRIMARY AMINE MIXTURES RELATIVE TO A FLUORESCAMINE–GLUTAMATE MIXTURE AT pH 7.6[a]

Amine	Relative fluoresence[b]		
	pH 5.8	pH 7.6	pH 9.2
pABA	102	100	102
$pABAglu_1$	103	105	105
$pABAglu_7$	98.4	94.0	98.8
o-Aminobenzoic acid	3.86	4.47	4.42
Aniline	47.1	58.2	41.5
Adenosine	0.0072	0.0072	0.0037
Alanine	0.013	0.42	1.21
Tryptophan	0.0076	0.22	2.10
Glutamate	0.022	1.00	2.67

[a] The amines were dissolved in either 0.1 M sodium acetate pH 5.8, 0.1 M potassium phosphate pH 7.6, or 0.1 M sodium phosphate pH 9.0 at various concentrations after which 15 μl of fluorescamine (3 mg/ml of dry acetone) was added and the fluorescence determined as described under Materials and Methods. All determinations were performed in triplicate and the values averaged. The maximum variation was ±3%.

[b] Excitation wavelength 396 nm, emission wavelength 495 nm.

orescamine in a direct assay of column eluates for $pABAglu_n$ avoiding the use of radioactive precursors.

MATERIALS AND METHODS

Fluorescamine, all other biochemicals, and column resins were obtained from Sigma. Bactopeptone, bactotryptone, yeast extract, and vitamin-free casamino acids were obtained from Difco. The γ-glutamylcarboxypeptidase was prepared from chicken pancreas as described by Bird *et al.* (2). Fluorescence measurements were carried out using an Aminco–Bowman spectrophotofluorometer. Amino acid analyses were carried out using a Technicon NC2P amino acid analyzer operated by the Department of Chemistry, University of Manitoba. Light absorbance measurements were carried out using a Beckman Acta III spectrophotometer.

Preparation of extracts. The following bacterial strains were grown in either LB medium (14) containing 5 g NaCl, 5 g yeast extract, and 10 g bactotryptone per liter (medium 1) or a minimal medium containing 0.1 M Tris pH 7.6, 10 g vitamin-free casamino acids, 5 g glucose, 5 g NaCl, 0.16 mM Na_2SO_4, 1 mM $MgSO_4$, 0.03 mM $FeSO_4$, 0.5 mM KH_2PO_4, and 0.2 mM $CaCl_2$ (medium 2): *Escherichia coli* B23 (wild type, this laboratory), *Staphylococcus aureus* ATCC 6538D, *Streptococcus faecalis* ATCC 8043, *Salmonella arizonae* ATCC 13314, *Bacillus subtilis* ATCC 6051, and *Lactobacillus casei* ATCC 393. The length of growth is specified in the figure legends. The cells were collected by centrifugation and washed once in 0.1 M potassium phosphate pH 7.6. The pellet was weighed, resuspended in 10 ml of the same buffer and boiled for 5 min. The debris was removed by centrifugation and the supernatant used to prepare the $pABAglu_n$ fraction for ion-exchange chromatography. The rat liver extracts were prepared by homogenizing 1 g of rat liver in 0.2 M HCl after which the debris was removed by centrifugation. The fresh water fungus *Achlya* sp. was grown to stationary phase in a medium containing 1 g peptone, 1 g yeast extract, and 3 g glucose per liter and was kindly supplied by Dr. G. Klassen. The cells were collected by filtration, washed with

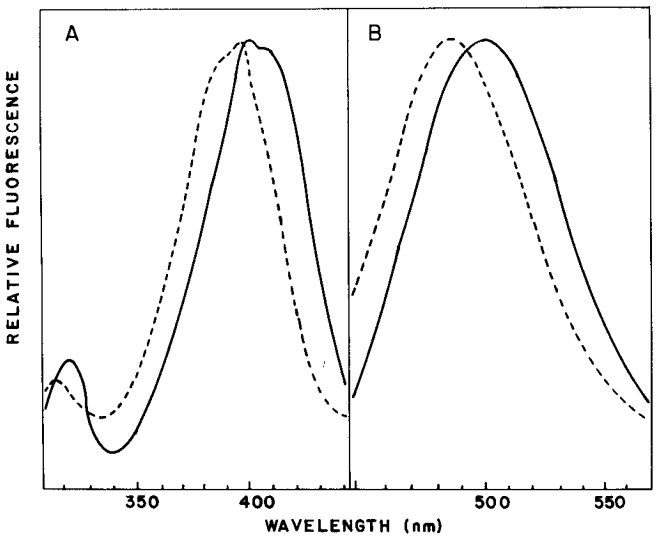

FIG. 1. Excitation (A) and emission (B) spectra of 0.01 mM pABA-fluorescamine (——) and of 1 mM glutamate-fluorescamine (- - -) in 0.05 M potassium phosphate pH 7.6. Both solutions were prepared by adding 15 μl of fluorescamine (3 mg/ml in dry acetone) to 1 ml of primary amine at room temperature.

water, and resuspended in 0.1 M potassium phosphate pH 7.6. After boiling for 5 min, the debris was removed by centrifugation. Commercial bakers yeast was resuspended in 0.1 M potassium phosphate pH 7.6, boiled for 5 min, and the debris was removed by centrifugation.

Preparation of the pABAglu$_n$ fraction for chromatography. After acidification to pH 1.0 using concentrated HCl, all extracts were treated as described by Shane (10) up to and including the treatment with zinc metal. One of the following two protocols was then followed in order to prepare the solution for ion-exchange chromatography.

1. The supernatant from zinc reduction was treated to convert the pABAglu$_n$ to their azo dye derivatives following the procedure of Shane (10). Isolation and cleavage of the azo dye derivatized pABAglu$_n$ was carried out following the outline of Brody (13). Specifically, 3 ml of Dowex AG50W-X8 (acid form) was added to the 20 ml solution of azo dye. After gently stirring for 15 min, the suspension was packed into a 1-cm diameter column and washed with 40 ml 0.2 M HCl, 10 ml H_2O, 5 ml 0.05 M potassium phosphate pH 7.6, and 5 ml H_2O. The azo dye was then reduced and the pABAglu$_n$ were eluted with 10 ml 0.3 M sodium hyposulfite. The purple color adhering to the Dowex resin disappeared upon addition of the sodium hyposulfite. Fractions were collected from which 10 μl was taken and added to 1 ml of 0.1 M potassium phosphate pH 7.6 for reaction with 15 μl of fluorescamine (3 mg/ml in dry acetone). Fluorescence was determined using an excitation wavelength of 400 nm and an emission wavelength of 500 nm. All material reacting with fluorescamine eluted as a single peak and these fractions were pooled and concentrated to dryness. The residue was resuspended in 10 ml of 0.04 M potassium phosphate pH 7.6, 0.15 M NaCl, and any precipitate was removed by centrifugation. The supernatant was charged onto a DEAE-Sephadex A25 column.

2. The second protocol bypasses the azo dye formation. The supernatant from the zinc treatment was neutralized with KOH, centrifuged to remove any precipitate and diluted to produce a KCl concentration of 0.15 M. This solution was charged directly onto a DEAE-Sephadex A25 column.

DEAE-Sephadex chromatography. The solutions prepared by either protocol were

charged onto 0.7 × 50-cm DEAE-Sephadex A25 columns equilibrated with 0.15 M NaCl in 0.04 M potassium phosphate pH 7.6. After being washed with 50 ml of the equilibrating buffer, the columns were eluted with a linear gradient prepared by mixing 250 ml of 0.15 M NaCl–0.04 M potassium phosphate pH 7.6 and 250 ml of 0.7 M NaCl–0.04 M potassium phosphate pH 7.6. Fractions of 2.5 ml were collected from which 1 ml was taken and mixed with 15 μl of fluorescamine (3 mg/ml of dry acetone). Fluorescence was determined as described above.

Characterization of polyglutamate chain length. Where further analysis was necessary, the relevant fractions were pooled and concentrated by evaporation. The residue was dissolved in 1 ml of H_2O and desalted by passage through a 1 × 100-cm column of Sephadex G10 eluted with 0.1 M triethylammonium bicarbonate pH 7.6. Material reacting with fluorescamine, which usually eluted in the void volume, was concentrated to dryness and redissolved in 1 ml of H_2O. The uv absorbance spectrum with and without 0.1 M HCl was obtained to confirm and quantitate the presence of pABA. Aliquots were hydrolyzed for amino acid analysis in a sealed tube with 6 N HCl at 100°C for 16 h. Partial hydrolyses were carried out in a sealed tube with 1 N HCl at 100°C for 45 min. Partial hydrolysis with γ-glutamylcarboxypeptidase was carried out as described by Bird *et al.* (2).

RESULTS

Relative fluorescence of the pABA–fluorescamine adduct. Fluorescamine reacts with a wide variety of primary amines to produce highly fluorescent adducts. Compiled in Table 1 are the fluorescence values of a number of these adducts relative to the fluorescence of the glutamate adduct at pH 7.6. Of the compounds tested, pABA and its polyglutamate derivatives formed adducts with the greatest relative fluorescence. Furthermore, the relative fluorescence of the pABA adduct did not change over the pH range 5.5 to 9.0, whereas the fluorescence of the amino acid adducts dropped as the pH was lowered. For comparison, the excitation and emission fluorescence spectra of the pABA and glutamate adducts are shown in Fig. 1. In addition to the enhanced fluorescence of the pABA adduct, its spectra also exhibited other differences including the shift to longer wavelength of the wavelengths of maximal excitation and emission. Fluorescamine has been found to be much more sensitive than ninhydrin as a probe for amino acids and the intense fluorescence of the pABA–fluorescamine adduct suggested that fluorescamine might also provide a sensitive probe for $pABAglu_n$ derived from folic acid.

Extraction of the $pABAglu_n$. The procedure described by Shane (10) for the extraction of folic acid and its subsequent cleavage to $pABAglu_n$ was used for all experiments. The two procedures which were used to prepare the $pABAglu_n$ for ion-exchange chromatography differed in whether or not the azo dye derivatives were formed. Using published procedures (8,10), greater than 98% of the pABA-containing material could be converted to azo dye. The subsequent binding of the azo dye to Dowex from which the $pABAglu_n$ were released by reduction using sodium hyposulfite had been outlined but not completely described by Brody (13). The protocol which was developed from this outline was reliable but the recovery was only 60–65%. No improvement in this recovery could be achieved despite several attempts in which known amounts of pABA, $pABAglu_1$, and $pABAglu_7$ were derivatized, bound to, and eluted from, Dowex under a number of different conditions. In bypassing the azo dye preparation and proceeding directly to load the $pABAglu_n$ solution on DEAE-Sephadex, this loss of material was avoided without any enhancement of background fluorescence in the elution pattern. The resulting elution patterns from both methods were identical except for the greater amount of material in the nonderivatized eluates.

Elution of $pABAglu_n$ from DEAE-Sephadex A25. Several reports have de-

scribed the use of DEAE-cellulose to separate pABAglu$_n$ mixtures (3–7) and the use of DEAE-Sephadex in this report for the same purpose represents a minor modification. To ensure reproducibility and eliminate the possibility of contaminating peaks, all resin was pretreated, first with 0.5 M HCl and then with 0.5 M KOH. The resin was washed with distilled water after each treatment and then equilibrated with 0.15 M NaCl, 0.04 M potassium phosphate pH 7.6. A new column was prepared and washed with equilibrating buffer for several hours for each determination. Packing and charging the column as well as assaying the eluate did not require more than 4 or 5 h spread over 2 or 3 days and the column elution took 36 h. Adaptation of high-pressure liquid chromatography would shorten the elution time and eliminate the necessity of column repacking.

It was possible to determine the amount of each pABAglu$_n$ in the extract directly from the peak area in the elution profile. The absolute fluorescence per mole of pABA (1.8 fluorescence units/1 nmol pABA) did not change over the course of the experiments providing that the sensitivity, slit, and blank adjustments on the spectrophotofluorometer were not changed. In order to confirm the validity of this procedure, 5 nmol of each of pABA, pABAglu$_1$, and pABAglu$_7$, both as a mixture and in individual experiments, were eluted from DEAE-Sephadex and quantitated. The poorest recovery as determined from the peak size was 4.81 nmol or 96% and normal recoveries were above 4.9 nmol. When the azo dye derivatization protocol was followed, it was necessary to take into consideration the 40% loss of material when calculating the amount of pABAglu$_n$.

Characterization of the pABAglu$_n$ species. The elution profiles of extracts from exponential and stationary phase *E. coli* are shown in Fig. 2a. Authentic pABA and pABAglu$_1$ eluted at the positions indicated by 0 and 1, and, while it was possible to directly read the chain length of the various pABAglu$_n$ from the elution profile, three further steps were carried out to confirm the assignments.

1. Treatment of any one of the peaks, including that assigned as pABAglu$_5$, with γ-glutamylcarboxypeptidase from chicken pancreas produced pABAglu$_2$ which eluted as shown in Fig. 2b.

2. Each of the peaks in the elution profiles was desalted and subjected to a partial acid hydrolysis after which the mixture was chromatographed on DEAE-Sephadex A25. The elution profile of partially hydrolyzed pABAglu$_8$ is also shown in Fig. 2b where there are clearly nine peaks corresponding to each size from pABA to pABAglu$_8$. Because of its lower relative fluorescence, the glutamate liberated in the reaction did not show up in the elution profile.

3. Each of the peaks in Figs. 2a and b was subjected to amino acid analysis and the glutamate-to-pABA ratio calculated using the molar absorptivity of pABA at 274 nm as 13.2×10^3 M^{-1} cm^{-1} at pH 7.6. These results are tabulated in Table 2.

The elution profiles of extracts from various other bacteria were also prepared. The composition of the extract of stationary phase *S. arizonae* was very similar to that of *E. coli* (Fig. 2c). The extracts of both exponential and stationary phase *S. faecalis* contained pABAglu$_4$ as the main species (Fig. 3a). Primarily pABAglu$_8$ with smaller amounts of pABAglu$_7$ and pABAglu$_9$ was found in an extract of *L. casei* (Fig. 3b). The extract of *S. aureus* contained principally pABAglu$_4$ whether from exponential or stationary phase cells (Fig. 3c), although there was a significant increase in the total amount of pABAglu$_n$ in the stationary phase cell extract (Table 3). Finally, *B. subtilis* isolated during exponential growth contained predominantly pABAglu$_3$ (Fig. 3d).

In order to show that the fluorescamine assay for pABAglu$_n$ was applicable to other than just bacterial extracts, baker's yeast, the freshwater fungus *Achlya* sp., and rat liver extracts were prepared and chromato-

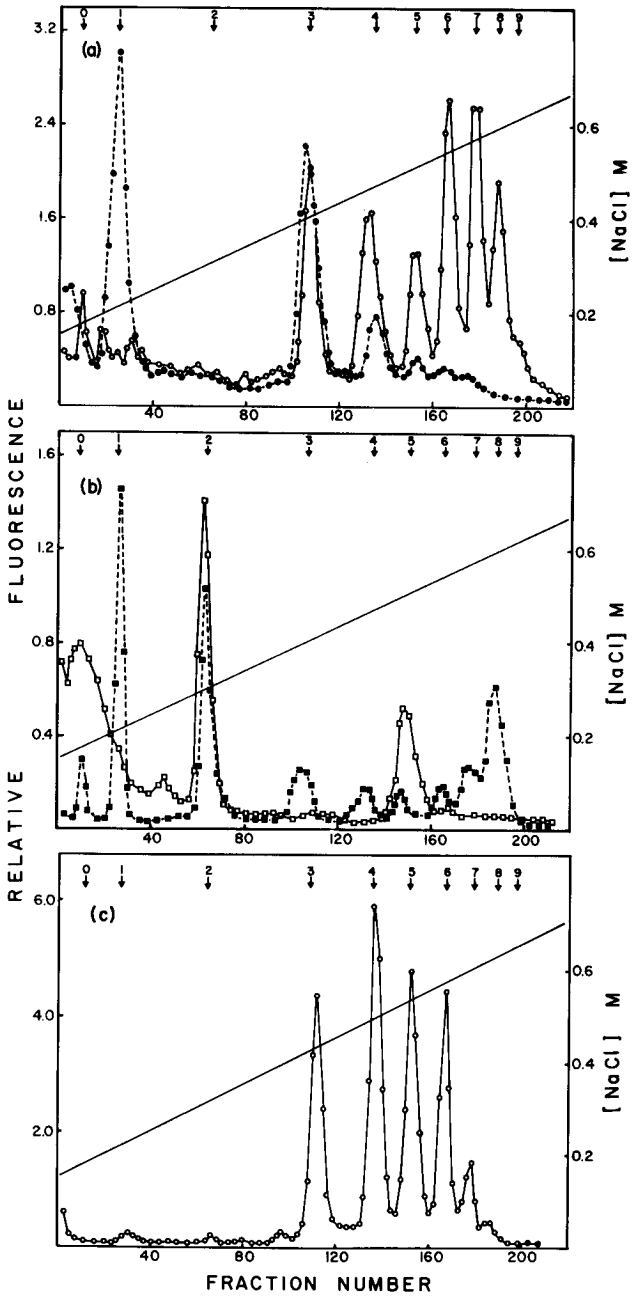

FIG. 2. (a) DEAE-Sephadex elution profiles of extracts of 3.2 g of exponential phase (● - - - ●) and 5.8 g of stationary phase (○ —— ○) *E. coli* grown in LB medium at 37°C for 4 and 16 h, respectively. (b) DEAE-Sephadex elution profiles of $pABAglu_8$ after partial acid hydrolysis (■ - - - ■) and of $pABAglu_5$ after partial degradation with γ-glutamylcarboxypeptidase (□ —— □). Both $pABAglu_5$ and $pABAglu_8$ were isolated from the eluate in a. (c) DEAE-Sephadex elution profile of an extract of 6.3 g of stationary phase *S. arizonae* grown in LB medium at 37°C for 16 h. The arrows indicate the elution positions of the different $pABAglu_n$.

TABLE 2

GLUTAMIC ACID: pABA RATIOS IN VARIOUS $pABAglu_n$ FRACTIONS SEPARATED IN FIGURE 2[a]

Peak	pABA[b] (nmol)	Glu[b] (nmol)	Glu:pABA
1	9.8	10.4	1.07
2	6.8	16.2	2.37
3	7.6	24.8	3.26
4	8.4	34.0	4.05
5	29.5	143.8	4.85
6	9.0	53.9	6.02
7	21.3	145.1	6.81
8	14.1	113.1	8.01

[a] The procedures for quantitation of pABA by uv absorbance and of glutamate by amino acid analysis are described in the methods and results sections.

[b] The variation in uv absorbance measurements was ±1% while the error in amino acid analysis determination was ±10%.

graphed on DEAE-Sephadex. The yeast extract contained mainly $pABAglu_7$ with smaller amounts of $pABAglu_6$ and $pABAglu_8$ (Fig. 4a). Mainly $pABAglu_5$ and $pABAglu_6$ were found in the rat liver extract (Fig. 4b) and a mixture of all possible pABA-polyglutamates from $pABAglu_1$ to $pABAglu_9$ was found in the extract of *Achlya* sp. (Fig. 4c).

DISCUSSION

The intense fluorescence of the fluorescamine–pABA adduct has been exploited in the development of a sensitive assay for pABA and $pABAglu_n$. The use of radioactive precursors was avoided eliminating any possibility of poor transport or poor metabolism of the precursor having an effect on the apparent composition of the *in vivo* folic acid pool. The assay was sufficiently sensitive that as little as 25 pmol/ml of pABA could be clearly detected, although, because of sample dilution during column elution, a minimum of 0.5 nmol of pABA was necessary for quantitation after ion-exchange chromatography. As a result, relatively small, easily prepared amounts of cell paste or tissue could be used to produce satisfactory elution profiles with the actual amount required depending on the folate content of the organism. *S. aureus* produced very large amounts of $pABAglu_n$-containing material, largely as a single species $pABAglu_4$, and less than 0.1 g of cell paste was sufficient, whereas 1–3 g of other bacteria were required because of lower folate levels and a broader distribution of glutamate chain length. On a routine basis, 1 g or more of cell paste or tissue was a good starting point for a first analysis of any organism. Adaptation of high-pressure liquid chromatograhy with a fluorescamine mixing chamber and fluorometer flow cell like that used in amino acid analyzers would significantly reduce the amount of sample required.

The reason for the more intense fluorescence of the pABA–fluorescamine adduct relative to other amino acid fluorescamine adducts was not investigated but two points suggested that an electron association between the aromatic portions of pABA and fluorescamine was involved. First, the wavelengths of maximal emission and excitation were shifted to longer wavelengths. Second, the lower fluorescence of the o-ABA adduct could be attributed to steric interference of the carboxylic acid adjacent to the amino group causing a change in the orientation of the two aromatic groups reducing the electron association. Of significance to the assay of $pABAglu_n$ in column eluates was the fact that no other naturally occurring primary amines capable of forming such intensely fluorescent adducts were retained on DEAE-Sephadex to interfere with the assay.

The utility of the fluorescamine assay procedure for $pABAglu_n$ was confirmed in the satisfactory comparison of the results obtained using fluorescamine with the results in earlier reports. Mixtures of $pABAglu_{1-5}$ with $pABAglu_4$ as the predominant species have been reported for *S. faecalis* and *fae-*

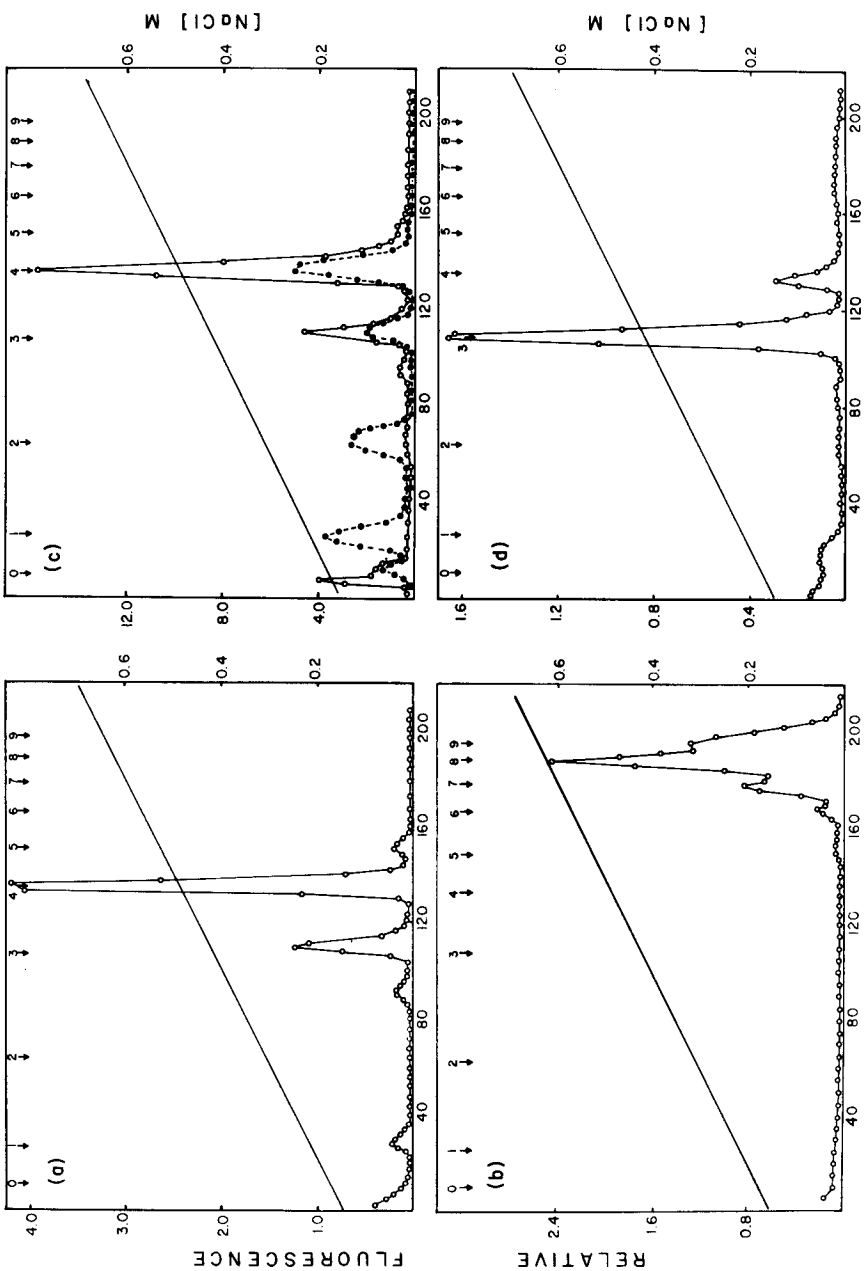

FIG. 3. (a) DEAE-Sephadex elution profile of an extract of 1.8 g of *S. faecalis* grown in LB medium at 37°C for 16 h. (b) DEAE-Sephadex elution profile of an extract of 1.6 g of *L. casei* grown in LB medium at 37°C for 40 h. (c) DEAE-Sephadex elution profiles of an extract of 0.7 g of *S. aureus* grown in LB medium at 37°C for 16 h (○——○) and of $pABAglu_4$ following partial acid hydrolysis (●- - -●). (d) DEAE-Sephadex elution profile of an extract of 1.8 g of *B. subtilis* grown in LB medium for 4 h at 37°C. The arrows indicate the elution positions of the different $pABAglu_n$.

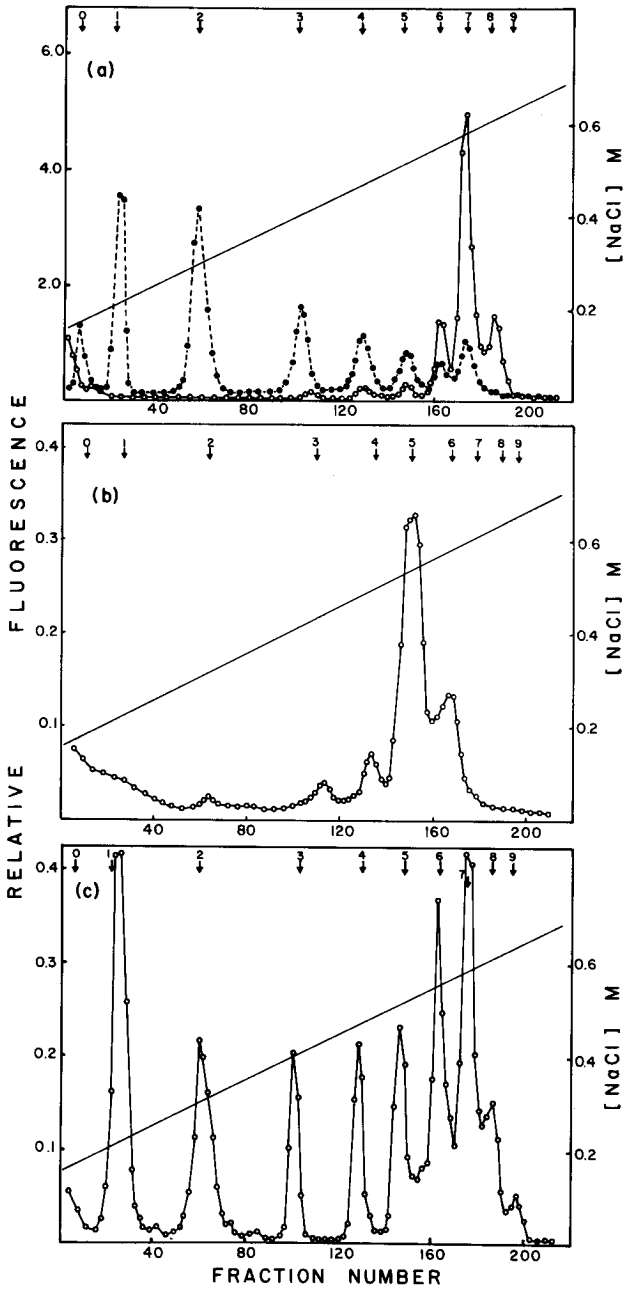

FIG. 4. (a) DEAE-Sephadex elution profiles of an extract of 2.0 g (wet weight) commercial baker's yeast (○——○) and of $pABAglu_7$ following partial and hydrolysis (●- - -●). (b) DEAE-Sephadex elution profile of an extract of 1.0 g of rat liver. (c) DEAE-Sephadex elution profile of an extract of 5.0 g *Achlya* sp. The arrows indicate the elution positions of the different $pABAglu_n$.

cium (4,5,8). For *E. coli*, exclusively $pABAglu_3$ (15), a mixture of $pABAglu_{3-5}$ (3,16), and a mixture of $pABAglu_{1-7}$ (17) have all been reported, most likely reflecting differences in growth phase at isolation. For *L. casei*, exclusively $pABAglu_8$ (8), a mix-

TABLE 3

AMOUNT OF pABAglu$_n$ PER GRAM OF CELL PASTE AS DETERMINED FROM THE PEAK SIZE IN FIGURES 2-4[a]

Cell extract	Nanomoles of pABAglu$_n$ per gram of cell paste									
	n: 1	2	3	4	5	6	7	8	9	Total
E. coli (exponential phase)	19.9	—	18.5	6.2	2.1	1.6	0.8	—	—	49.1
E. coli (stationary phase)	—	—	10.2	11.9	7.7	13.0	13.3	9.4	1.7	67.2
S. arizonae (stationary phase)	—	tr	14.4	22.2	15.8	13.0	3.2	0.8	—	69.4
S. faecalis	—	0.8	13.4	56.4	0.8	—	—	—	—	71.4
L. casei	—	—	—	—	tr	1.1	5.4	16.6	4.0	27.1
S. aureus (exponential phase)	—	—	10.0	40.4	—	—	—	—	—	50.4
S. aureus (stationary phase)	tr	2.0	115.8	473.0	tr	—	—	—	—	590.8
B. subtilis	—	—	31.6	2.4	—	—	—	—	—	34.0
Baker's yeast	—	tr	1.0	1.8	1.8	10.2	44.4	9.8	—	69.0
Rat Liver	—	tr	1.2	5.0	22.5	8.6	—	—	—	37.3
Achlya sp.	3.2	0.8	0.8	0.8	0.8	1.2	2.2	0.4	tr	10.2

[a] The total amount of fluorescence in each peak in Figs. 2-4 was determined and equated to nanomoles pABA using the relationship 1 nmol/ml pABA gives 1.8 fluorescence units/ml.

ture of pABAglu$_{1-6}$ (4), and a mixture of pABAglu$_{7+}$ (3) have all been reported and the variations have been attributed to differences in folic acid concentration in the growth medium. Exclusively pABAglu$_5$ (5) and a mixture of pABAglu$_{4-6}$ (7) have been reported for rat liver and a mixture of pABAglu$_{6-8}$ with pABAglu$_7$ as the predominant species has been reported for yeast (6). The distribution of pABAglu$_n$ in *B. subtilis* and *S. aureus* have not been reported but the predominance of the tri- and tetraglutamate forms is similar to the results in most other bacteria.

Earlier reports had noted that the folic acid concentration in the growth medium had a significant effect on the polyglutamate chain length in *L. casei* (5,8). The change from exponential to stationary growth phase in *Escherichia coli* elicited a similar response resulting in the polyglutamate chain length increasing during the transition to stationary phase. Unlike the change in *L. casei*, this change was independent of folic acid concentration in the medium, being observed in both LB medium and in minimal medium (data not shown). Excepting *S. arizonae* the pABAglu$_n$ chain length in the other bacteria studied in this report were unaffected by the transition to stationary phase. Any explanation for these changes in polyglutamate chain length must await a more detailed analysis of folate metabolism in *E. coli*.

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