Proc. Natl. Acad. Sci. USA
Vol. 78, No. 5, pp. 2869–2873, May 1981
Biochemistry

Identification of hypusine, an unusual amino acid, in a protein from human lymphocytes and of spermidine as its biosynthetic precursor

[N^6-(4-amino-2-hydroxybutyl)-2,6-diaminohexanoic acid or N^ϵ-(4-amino-2-hydroxybutyl)lysine/putrescine/polyamines/posttranslational modification]

MYUNG HEE PARK*, HERBERT L. COOPER†, AND J. E. FOLK*

*National Institute of Dental Research, and the †National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Communicated by John M. Buchanan, February 9, 1981

ABSTRACT **When normal human peripheral lymphocytes are treated with mitogen and grown in the presence of [^{3}H]putrescine or [*terminal methylenes*-^{3}H]spermidine, label is incorporated predominantly into one cellular protein. The radioactive constituent of this protein was identified as the unusual amino acid hypusine [N^ϵ-(4-amino-2-hydroxybutyl)lysine]. This was accomplished by isolation of the component from proteolytic digests or acid hydrolysates and comparison with authentic hypusine by chromatography, conversion to the 2,4-dinitrophenyl derivative, and oxidative degradation. The observed relationships among intracellular levels of labeled putrescine, polyamines, and protein bound hypusine after growth of cells with the various labeled amines and with or without an inhibitor of polyamine biosynthesis supply evidence that spermidine is the immediate amine precursor of hypusine and that the 4-amino-2-hydroxybutyl portion of hypusine derives from the butylamine moiety of spermidine.**

While studying the role of polyamines as physiological substrates for transglutaminases, we observed an unidentified radiolabeled material in proteolytic digests of the protein fraction from normal human peripheral lymphocytes that had been treated with mitogen in the presence of [^{3}H]putrescine. This labeled basic component of lymphocyte protein was initially thought to be N^1-(γ-glutamyl)spermidine because it chromatographed in a position close to that of this γ-glutamylpolyamine in the ion exchange system used. It was subsequently recognized and reported (1) as an unknown component of lymphocyte protein. This conclusion was based on its observed stability to acid hydrolysis. We now report that this compound, which is the major labeled component formed in the lymphocyte protein fraction during growth of the cells with either labeled putrescine or labeled spermidine, is the unusual amino acid hypusine. The present report provides evidence for the identity of this amino acid and for the direct precursor role of spermidine in its biosynthesis. In addition, it is shown that hypusine occurs predominantly in one protein of lymphocytes.

Hypusine was discovered by Nakajima and coworkers (2), who isolated the free amino acid from homogenates of bovine brain and determined its structure as N^ϵ-(4-amino-2-hydroxybutyl)lysine. In addition to its detection in free form in a number of organs of several mammals (3), hypusine was found to be a constituent of animal proteins (4). Attempts to find any specific protein abundant in this amino acid were, however, without success (4).

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EXPERIMENTAL PROCEDURE

Materials. [2,3-^{3}H(N)]Putrescine·2HCl, [*terminal methylenes*-^{3}H(N)]spermidine·3HCl, and [3-*aminopropyl*-3-^{3}H(N)]-spermine·4HCl were purchased from New England Nuclear. Their specific radioactivities were >20 Ci/mmol (1 Ci = 3.7 × 10^{10} becquerels) and each was used without dilution of specific radioactivity. Methylglyoxal bis(guanylhydrazone)·2HCl·H_2O (MGBG) was from Aldrich. Hypusine, isolated by T. Nakajima and his colleagues and provided by them to John W. Daly of the National Institutes of Health, was a gift from him to us. Other materials and reagents have been described (1).

Methods. Human peripheral lymphocytes from healthy normal donors were purified by a combination of nylon column adsorption and density separation as described (5) and cultured at a density of 10^6 cells per ml in RPMI 1640 containing glutamine (0.3 mg/ml), penicillin (50 international units/ml), streptomycin (50 international units/ml), 10 mM Hepes, pH 7.4, and 10% autologous plasma. Growth was induced by addition of phytohemagglutinin (Wellcome, Beckenham, England) at 5 μg/ml the day after purification. Additions of labeled amines were made together with the mitogen at 5 μCi/ml except where noted otherwise. Cells were harvested by centrifugation for 10 min at 260 × *g* at 35°C. The cells were washed twice with ice-cold phosphate-buffered saline (pH 7.4). The cell pellet was suspended in a small volume of phosphate-buffered saline, and an equal volume of 20% trichloroacetic acid was added. Further details of preparation of trichloroacetic acid precipitates and supernatant fractions are given elsewhere (1). Enzymic digestions of trichloroacetic acid precipitates were carried out as described (1). Acid hydrolysis was performed in 6 M HCl at 106°C for 18 hr in sealed evacuated tubes.

Ion exchange chromatographic separations were conducted by using a Dionex D-400 analyzer with the three-buffer system under conditions described (1). For measurement of radioactivity and other external analyses, collection of effluent was made directly from the column without mixing with fluorometric reagent.

Radioactivity was measured in Hydrofluor counting fluid (National Diagnostics, Somerville, NJ) by using a liquid scintillation spectrometer.

RESULTS

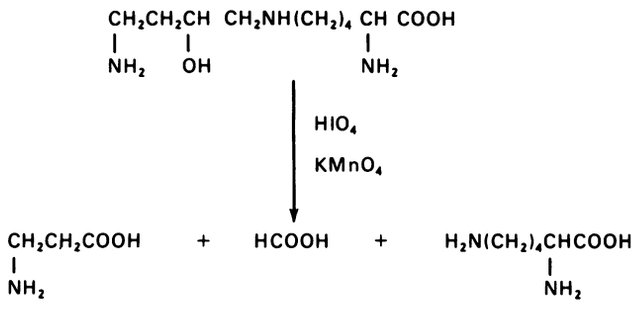
Identification of Hypusine in the Protein Fraction of Lymphocytes. Peak I of Fig. 1 shows the position in ion exchange

Abbreviations: MGBG, methylglyoxal bis(guanylhydrazone); DNP, 2,4-dinitrophenyl.

chromatography of the major radioactive compound formed by acid hydrolysis of the protein fraction from lymphocytes grown in the presence of [^{3}H]putrescine. A peak of radioactivity is found in the same position after chromatography of an acid hydrolysate of the protein from cells grown with [*terminal methylenes*-^{3}H]spermidine in place of [^{3}H]putrescine. Exhaustive proteolytic digests prepared from the protein fractions of cells grown with each of the labeled amines show patterns of radioactivity similar to that shown in Fig. 1. Typical chromatographic patterns of radioactivity in proteolytic digests are given elsewhere (see figure 2 of ref. 1). Peaks II and III of Fig. 1 are those of standard [^{3}H]N^1-(γ-glutamyl)spermidine and N^8-(γ-glutamyl)spermidine, respectively, chromatographed in this system. Comparison of the elution profiles of the labeled compounds with those of standard nonlabeled hypusine, N^1-(γ-glutamyl)spermidine, and N^8-(γ-glutamyl)spermidine (Fig. 1, *Inset*; elution times of 1638 sec, 1694 sec, and 1764 sec, respectively) shows that the acid-stable labeled compound from lymphocyte protein chromatographs in the position of hypusine in this system.

Table 1 summarizes the results of thin-layer chromatography of the 2,4-dinitrophenyl (DNP) derivatives of hypusine and the acid-stable radioactive component of lymphocyte protein. That the two derivatives chromatograph in identical positions in several solvent systems is substantial evidence for the identity of the lymphocyte component as hypusine.

Further evidence for the identity of this component was obtained from oxidation experiments, the results of which are also given in Table 1. Nakajima and coworkers (2) showed that oxidation of hypusine with periodate cleaves the molecule at the vicinal amino alcohol group and that the products obtained after further treatment with permanganate are β-alanine, formic acid, and lysine:



When the radioactive component of protein from cells grown with either [^{3}H]putrescine or [*terminal methylenes*-^{3}H]spermidine was oxidized in this manner and the products were dinitrophenylated and examined by thin-layer chromatography, radioactivity was found only in the position of DNP-β-alanine in several solvent systems; no label appeared in the α,ε-diDNP-lysine region. Consistent with this finding was that obtained by ion exchange chromatography of samples of oxidation mixtures that had not been dinitrophenylated. With oxidation mixtures of the radioactive component from cells grown in the presence of either of the amines, label was found to elute in the position of β-alanine. No label, however, appeared in the lysine position. The presence of labeled β-alanine in these oxidation mixtures is additional evidence that the labeled amino acid component of lymphocyte protein is hypusine. Because no label is found in lysine after oxidation, it may be concluded that only the 4-amino-2-hydroxybutyl moiety of the hypusine in lymphocyte protein contains radioactivity derived from putrescine or spermidine.

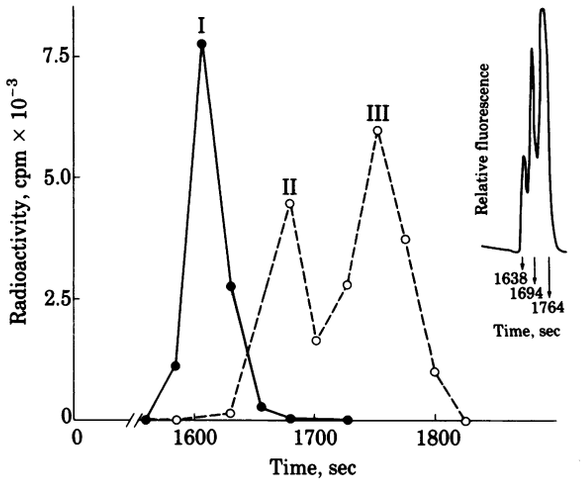


FIG. 1. Ion exchange chromatographic separation of the labeled acid-stable component of lymphocyte protein (I) from N^1-(γ-glutamyl)spermidine (II) and N^8-(γ-glutamyl)spermidine (III). The protein fraction was prepared from lymphocytes (1.5×10^7) that had been incubated with mitogen and [^{3}H]putrescine for 24 hr. The chromatographic conditions are those given in the inset to figure 2 of ref. 1. Fractions were collected every 24 sec. Preparation of the ^{3}H-labeled γ-glutamylspermidines was carried out as described (1), except for the use of ^{3}H-labeled polyamine. (*Inset*) Recorded elution of standard nonlabeled hypusine, N^1-, and N^8-(γ-glutamyl)spermidines, respectively, in the same chromatographic system with use of automatic fluorometric detection.

Identification of Spermidine as the Precursor of Hypusine. The data shown in Fig. 2 provide evidence that spermidine is the direct precursor of the 4-amino-2-hydroxybutyl portion of lymphocyte hypusine. Examination of the distribution of label in the cellular amine fractions from lymphocytes shows that, after 24 hr, more than half of the label in this fraction from cells grown with [^{3}H]putrescine is in the form of spermidine and spermine (experiment 1). This fraction from cells supplied with labeled spermidine, on the other hand, contains most of the label as spermidine and spermine and very little in the form of putrescine (experiment 3). This observation, together with the fact that both labeled putrescine and labeled spermidine are good exogenous sources of label for hypusine, suggests that spermidine or spermine, rather than putrescine, is the direct precursor of hypusine.

When lymphocytes are grown with [^{3}H]putrescine in the presence of MGBG, a potent inhibitor of the enzyme *S*-adenosylmethionine decarboxylase (7), most of the label in the cells is present as putrescine; little label occurs in the polyamines (experiment 2). This is the case because *S*-adenosylmethionine decarboxylase catalyzes formation of decarboxylated *S*-adenosylmethionine, which is the source of the propylamine moiety for the biosynthesis of spermidine from putrescine and of spermine from spermidine (for review, see refs. 8–10). The cells grown with [^{3}H]putrescine in the presence of MGBG also incorporate very little label into hypusine (experiment 2). In contrast, addition of MGBG with labeled spermidine (experiment 4) does not reduce the labeling of hypusine as compared with that which occurs without the inhibitor (experiment 3). As expected, MGBG does inhibit label from entering spermine. Consequently, most of the label in the amine pool of MGBG-treated cells that are supplied labeled spermidine remains as spermidine. The relationships between the labeled spermidine composition of the cellular amine pools and the labeled hypusine content provide strong evidence that hypusine in lymphocyte protein derives directly from cellular spermidine.

The results shown in Fig. 3 supply evidence that only label

Table 1. Identification of hypusine by thin-layer chromatography of its DNP derivative and the DNP derivatives of its oxidation products

Compound	R_F in solvent*			
	1	2	3	4
β-Alanine	0.65	0.7	0.85	0.5
Lysine	0.39	0.41	0.58	0.08
Hypusine				
Before oxidation	0.25	0.16	0.22	0
After oxidation	0.39, 0.65	0.41, 0.7	0.58, 0.85	0.08, 0.5
Radioactive material from cells treated with [2,3-^{3}H]putrescine				
Before oxidation	0.25	0.16	0.22	0
After oxidation	—	0.7	0.85	0.5
Radioactive material from cells treated with [*terminal methylenes*-^{3}H]spermidine				
Before oxidation	0.25	0.16	0.22	—
After oxidation	—	0.7	0.85	0.5

Cells were incubated for 24 hr with mitogen and ^{3}H-labeled putrescine or spermidine. Ion exchange chromatography of an acid hydrolysate of the trichloroacetic acid-insoluble fraction from these cells was carried out as outlined in the legend to Fig. 1. The fractions of labeled material eluted from the ion exchange column at the position of hypusine were combined and taken to dryness. The residue was dissolved in water, and the pH was adjusted to 7–7.5 with NaOH. After adjustment of the volume of solution to 0.3 ml with water, a portion containing $\approx 15 \times 10^3$ cpm was removed and dinitrophenylated directly by a published procedure (6). The remainder of the sample, containing $30–40 \times 10^3$ cpm, was made 1 M in NaOH, and 5 μl of benzoyl chloride was added. After vigorous stirring, the solution was allowed to stand for 30 min, at which time a second 5-μl portion of benzoyl chloride was added with stirring. After an additional 30 min, the mixture was made 1 M in HCl, and the benzoylated amino acid was separated from inorganic salts by extraction with three portions of ether. The combined ether extracts were taken to dryness, and to the residue was added 0.5 ml of 6 M HCl. After hydrolysis, the acid was removed under reduced pressure, and the residue was dissolved in water. Benzoic acid was extracted with ether and the free amino acid in the aqueous layer was oxidized with HIO_4 and $KMnO_4$ according to the published procedure (2).† The products of oxidation were dinitrophenylated (6). Location of radioactivity on thin-layer chromatograms was made by removing 2- to 3-mm segments of the layer along the development track and examining each for radioactivity in liquid scintillation fluid.

* Chromatography was carried out on silica gel G (Merck) in 1, chloroform/methanol/acetic acid (95:5:1); 2, chloroform/benzyl alcohol/acetic acid (70:30:3); 3, chloroform/*tert*-amyl alcohol/acetic acid (70:30:3); 4, benzene/pyridine/acetic acid (80:20:2).

† It was necessary to isolate the hypusine free of chromatographic reagents to attain satisfactory oxidation. This was accomplished by the benzoylation procedure.

from the butylamine moiety of the unsymmetrical polyamine, spermidine, enters hypusine during its biosynthesis. When lymphocytes were grown in the presence of spermine that was ^{3}H-labeled in only its propylamine portions, a significant degree of conversion of labeled spermine to spermidine occurred (experiment 3). However, no trace of radioactivity was found in the hypusine fraction from these cells. In parallel experiments with [^{3}H]putrescine, from which spermidine labeled in its butylamine moiety is formed (experiment 1), and with spermidine, in which both the butylamine and the propylamine portions are labeled (Experiment 2), good labeling of hypusine was observed. The simplest explanation for the failure of label to enter hypusine from labeled spermidine formed from [*3-aminopropyl*-3-^{3}H]spermine (experiment 3) is that this spermidine is labeled at the terminal methylene position of its propylamine moiety only and that ^{3}H in this position is not incorporated into hypusine during its biosynthesis. Indeed, there is evidence that the *in vivo* conversion of spermine to spermidine proceeds by way of direct oxidative cleavage of spermine to spermidine and 3-aminopropionaldehyde (11). The suggestion that label from the butylamine portion of spermidine, but not that from the terminal methylene position of its propylamine part, enters hypusine is consistent with the evidence from the oxidation experiments that only the 4-amino-2-hydroxybutyl moiety of hypusine contains label derived from spermidine.

Evidence that Hypusine Is Predominantly in One Lymphocyte Protein. When the proteins from lymphocytes grown with either [^{3}H]putrescine or [*terminal methylenes*-^{3}H]spermidine were analyzed by two-dimensional electrophoresis in polyacrylamide gels, a single strongly labeled protein of $M_r \approx 18,000$ and with a relatively acidic pI was detected (A in Fig. 4). In cells from this donor, a weakly labeled low molecular weight protein with a more basic pI may be seen (B in Fig. 4), but this was only apparent after 10 days to 2 weeks of fluorographic exposure. In repeated studies with lymphocytes from different normal donors, two small labeled proteins with relatively basic pIs were sometimes seen, as was reported earlier (1). However, only protein A was labeled in preparations from all donors, and it was the predominant one in four of five donors. Whether this variability is a result of differences among normal donors or is due to technical effects is not known.

Careful comparison of the stained gel of Fig. 4 and the fluorogram prepared from this gel indicates that protein A can be detectable by Coomassie blue staining. If this indeed is the case, protein A is not a minor species; the staining technique used detects only the more abundant proteins (12).

To define the radioactive constituent of the most intensely labeled protein (A in Fig. 4) as hypusine, we excised the areas containing this labeled protein from gels prepared from two separate donors, directly hydrolyzed each in acid, and analyzed each for hypusine. A single peak of radioactivity that eluted at the exact time of hypusine in the ion exchange chromatographic system of Fig. 1 was found in each case. Of the total trichloroacetic acid-precipitable radioactivity applied to the gels the fact that 35–40% was recovered from this labeled area of the gels is further evidence that this protein is the predominant hypu-

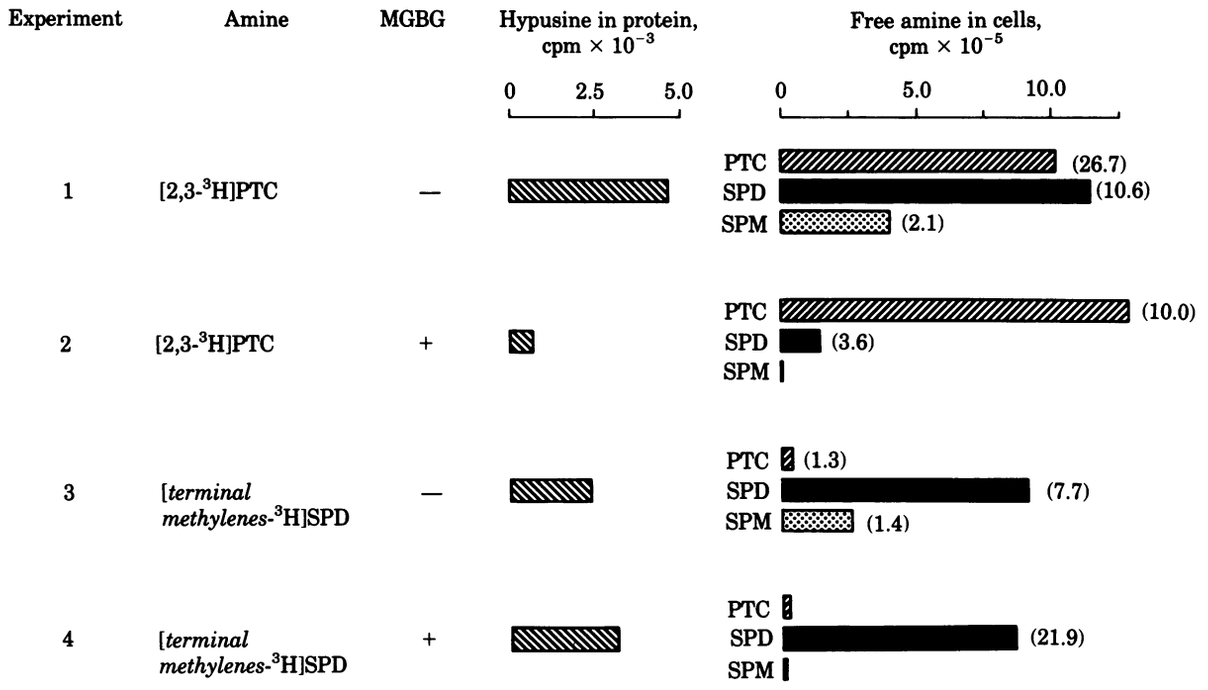


FIG. 2. Incorporation of radioactivity from putrescine and spermidine into hypusine in the protein fraction of growing lymphocytes; effects of MGBG. Experiments were conducted in parallel using cells from a single donor. Cells were cultured for 24 hr with mitogen and labeled amine with or without 10 μM MGBG. The trichloroacetic acid precipitates prepared from the cells were hydrolyzed with HCl, and the radioactivity in the hypusine fractions was measured after ion exchange chromatography. Putrescine, spermidine, and spermine in the trichloroacetic acid supernatants were separated by ion exchange chromatography and quantitated fluorometrically (1). A separate portion of supernatant was chromatographed for determination of radioactivity in the amines. Measurements of radioactivity refer to 5×10^6 cells. Numbers in parentheses at the ends of the amine bars are specific radioactivities in cpm $\times 10^{-5}$ per nmol of amine. PTC, putrescine; SPD, spermidine; SPM, spermine.

sine-containing protein of lymphocytes.

DISCUSSION

The data presented here provide evidence that hypusine is a constituent amino acid of a small acidic protein of lymphocytes and that the 4-amino-2-hydroxybutyl moiety of this unusual amino acid derives in part from the butylamine portion of spermidine. In preliminary experiments in which resting lymphocytes were incubated for 24 hr with [*terminal methylenes*-^{3}H]spermidine, little label was found in hypusine, even though the level of free labeled spermidine in these cells was high after the incubation. This failure to detect a significant degree of labeling of hypusine in resting cells may imply an essential role for the hypusine-containing protein in growing cells. It is important to determine whether the catalytic events that lead to production of hypusine occur subsequent to polypeptide chain assembly and, if so, whether the low level of labeling in resting cells reflects a lack of the protein substrate or inactivity of the

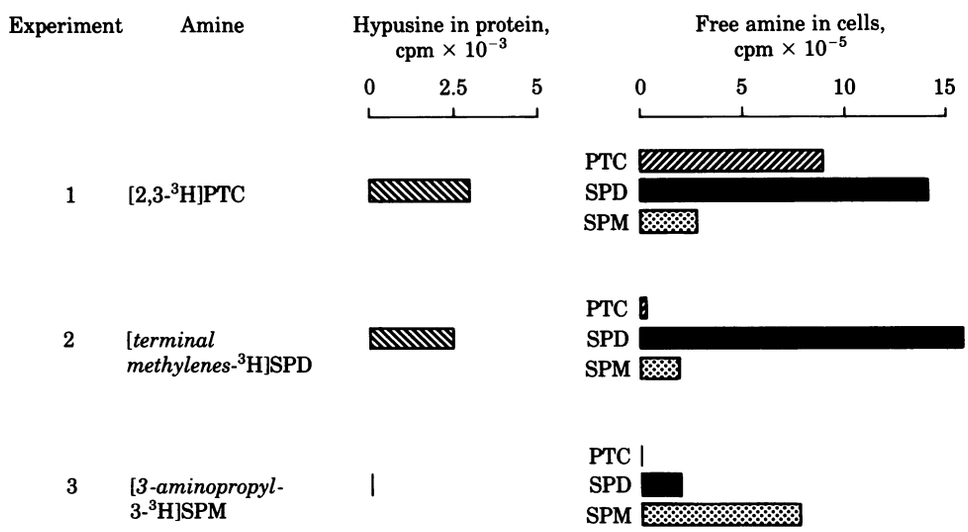


FIG. 3. Incorporation of radioactivity from putrescine, spermidine, and spermine into hypusine in the protein fraction of growing lymphocytes. Experiments were conducted in parallel using cells from a single donor. Cells were cultured for 24 hr with mitogen and labeled amine. Radioactivity in the hypusine and amine fractions was determined as outlined in the legend to Fig. 2. Measurements of radioactivity refer to 5×10^6 cells. The abbreviations are those of Fig. 2.

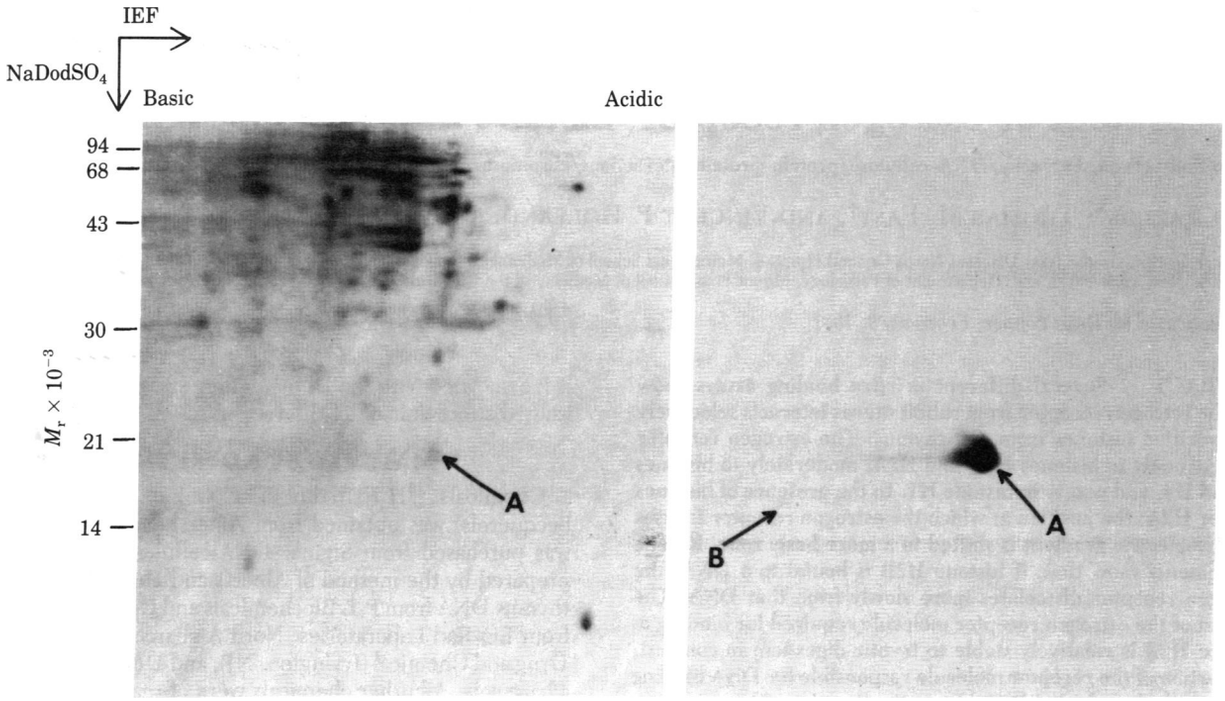


FIG. 4. Two-dimensional polyacrylamide gel separation of lymphocyte proteins. Cells were grown for 48 hr with mitogen and [^{3}H]putrescine at 10 μCi/ml. The sample was prepared for electrophoresis as outlined earlier (1) and an aliquot containing 80,000 cpm was used for preparation of the gel by the method of O'Farrell (12). The second dimension was NaDodSO$_4$/15% polyacrylamide. (*Left*) Pattern of protein staining with Coomassie blue. (*Right*) Fluorogram prepared from the same gel. Fluorography was performed as outlined (13) and exposure was to Kodak XR-5 film for 14 days. The protein designated A in the stained gel is that which was found exactly superimposible with the major radiolabeled protein designated A in the fluorogram. IEF, isoelectric focusing.

enzymic machinery required to catalyze the modification. The ultimate, but perhaps more difficult, goal will be the assignment of a role in cellular function to the hypusine-containing protein.

We recently reported that small amounts of labeled putrescine and spermidine are bound covalently to protein through γ-glutamylamide linkage in lymphocytes during growth with [^{3}H]putrescine (1). Whether our present failure to detect putrescine- and spermidine-containing proteins after two-dimensional gel electrophoresis is a consequence of distribution of the labeled amines in a number of proteins or is simply due to the relatively low amounts of labeled amines associated with one or a few proteins is not known. The amounts of radioactivity in the weakly labeled proteins observed after separation of some cell preparations were too small to allow identification of the labeled constituent.

There has been an accumulation of evidence for posttranslational modifications involving the ε-amino groups of lysine residues. Among these are production of ε-methylated lysine residues (14–17) and formation of ε-(γ-glutamyl)lysine crosslinks (for review, see ref. 18). In this regard, a suggestion of Imaoka and Nakajima (4) that hypusine is derived from protein-bound lysine by addition of a 4-amino-2-hydroxybutyl moiety at the lysine ε-amino group seems attractive. However, definitive experimental evidence has not as yet been offered in support of this hypothesis.

The recognition of spermidine as the precursor of the 4-amino-2-hydroxybutyl radical for hypusine biosynthesis adds new considerations to polyamine metabolic transformations. This finding, together with our recent observations on the transglutaminase-catalyzed incorporation of putrescine and polyamines *per se* into proteins in biological systems (1), reveals a structural contribution by these low molecular weight substances to biological macromolecules. Clues as to the catalytic mechanism by which spermidine contributes its butylamine moiety to the hydroxylated butylamine portion of hypusine do not appear to be contained in present knowledge of polyamine metabolism in mammals. Investigations of this transformation should, therefore, provide new insights into polyamine metabolic pathways.

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