Biochemical Pharmacology, Vol. 33, No. 5, pp. 779–785, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

HYDRALAZINE INHIBITION OF THE POST-TRANSLATIONAL HYDROXYLATION OF DEOXYHYPUSINE, A POLYAMINE-DERIVED AMINO ACID*

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(*Received* 6 *May* 1983; *accepted* 24 *August* 1983)

Abstract—Logarithmically growing Chinese hamster ovary cells, cultured in the presence of [1,4-^{14}C]putrescine, synthesize a protein(s) containing the unusual amino acid hypusine [*N*ε-(4-amino-2-hydroxybutyl)lysine]. This protein was separated and identified by two-dimensional gel electrophoresis and fluorography. The labeled hypusine isolated from an acid hydrolysate of the cell protein by ion exchange chromatography was identified by oxidative degradation and analyses of the products. Hydralazine, one of the most frequently prescribed drugs for the treatment of moderate to severe hypertension, added to the culture, resulted in the accumulation of a protein(s) containing the precursor amino acid deoxyhypusine [*N*ε-(4-aminobutyl)lysine]. Demonstration of this intermediate and its subsequent conversion to hypusine suggests that the synthesis occurs in several steps, one of these involving a hydroxylation reaction which can be inhibited by hydralazine.

The unusual amino acid hypusine [*N*ε-(4-amino-2-hydroxybutyl)lysine], discovered by Shiba *et al.* [1], results from the post-translational modification of lysine in peptide linkage [2]. Recently, Park *et al.* [3] found hypusine in a single labeled protein synthesized in phytohemagglutinin-stimulated lymphocytes cultured in the presence of either tritiated putrescine or spermidine. The protein containing hypusine could not be demonstrated in resting lymphocytes. Our laboratory confirmed the synthesis of hypusine containing protein in mitogen-stimulated lymphocytes and also found that this protein is synthesized by logarithmically growing Chinese hamster ovary cells and by normal diploid human lung fibroblasts (IMR 90) [4, 5]. Spermidine is the immediate precursor of hypusine as was initially reported [3], and the results to be presented here, as well as those of Park *et al.* [2], indicate that hypusine synthesis occurs in at least two steps. In the first step, the butyl or butylamine portion of spermidine is transferred to lysine in peptide linkage. Whether or not the ε-amino group of the lysine residue is retained and butylated, or displaced by butylamine, is as yet unknown. In the second step, the deoxyhypusyl intermediate formed is β-hydroxylated in a reaction which may be analogous to prolyl and lysyl residue hydroxylation of the nascent procollagen chains. Prolyl and lysyl hydroxylases belong to the group of mixed function oxidases which are α-ketoglutarate and Fe^{2+} dependent [6]. A reducing agent is also required, and ascorbic acid appears to be the most effective mediator [6, 7]. A few years ago we demonstrated that hydralazine, a commonly employed antihypertensive agent, could inhibit the hydroxylation of prolyl residues of procollagen synthesized by lung diploid fibroblasts in tissue culture [8]. Hydralazine has also been shown to inhibit dopamine β-hydroxylase [9].

In the present paper, we demonstrate that in cell culture hydralazine had a potent inhibitory effect on the hydroxylation reaction that leads to the synthesis of hypusine. This inhibition resulted in the accumulation of deoxyhypusyl residues which, when the cells were washed free of the drug and reincubated, were hydroxylated. In addition, hydralazine had an inhibitory effect on DNA synthesis and cellular growth and, to a lesser degree, on protein synthesis. Although the precise function of the hypusine-containing protein(s) is as yet unclear, experiments in progress suggest that this novel protein(s) may play a role in cellular growth.

MATERIALS AND METHODS

Chinese hamster ovary cells (CHO cells) were provided by Dr. Sam Latt from Children's Hospital in Boston. Hydralazine was a gift from CIBA, Summit, NJ. RPMI 1640 and the horse serum mycoplasma tested were obtained from GIBCO Laboratories, Grand Island, NY. [1,4-^{14}C]putrescine, sp. act. 102 mCi/mmole, was obtained from the New England Nuclear Corp., Boston, MA. *o*-Phthalaldehyde–Fluoropa (Dionex) was obtained from

* This work supported by National Institutes of Health Grants AG00376 and HL20764.

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the Pierce Chemical Co., Rockford, IL, and octane sulfonate monohydrate from the Aldrich Chemical Co., Milwaukee, WI. The polyamine standards, putrescine, spermidine, spermine (hydrochloride salts) and 1,7-diaminoheptane (base), were from the Sigma Chemical Co., St. Louis, MO. AG50W-X8 (100–200 mesh) was from BioRad, Richmond, CA. Chemicals were of the best quality available.

CHO cells were cultured in RPMI 1640 medium supplemented with 15% horse serum. After trypsinization, the cells were seeded at 2×10^6 cell density per T150 flask in the above medium and were incubated for 24 hr. The medium was then changed to fresh medium containing 15% heat-inactivated, extensively dialyzed horse serum. To determine the hydralazine dose/response curve, the following hydralazine concentrations were added to the medium: 0.3 mM, 0.2 mM, 0.1 mM and 0.05mM. The drug was omitted in the control cultures. Two hours later, [1,4-^{14}C]putrescine was added at a concentration of 0.25 μCi/ml, and the cells were incubated for 24 hr. In some experiments, the cells were incubated for 48 hr after the addition of [^{14}C]putrescine without changing the medium or with a change at 24 hr to a fresh medium that did not contain [^{14}C]putrescine or hydralazine. All the cultures were processed similarly at 4° as follows: the medium was separated from the cells and the cells were washed five times with cold phosphate-buffered solution. They were harvested with a rubber scraper in approximately 1 to 1.5 ml of phosphate-buffered solution per T150 flask. After sonication using 4 pulses of 5 sec each in a HEAT Systems-Ultrasonics, Inc. sonicator at 40% duty cycle, the samples were pooled, and aliquots were separated for DNA and protein determinations and two-dimensional gel electrophoresis. Protein was precipitated from the remaining cell sonicate with cold perchloric acid at a final concentration of 1.5 M. After standing at 4° overnight, the tubes were centrifuged at 900 *g*. The supernatant fraction was separated from the pellet and was processed as described below. The pellet was washed five times with cold 0.5 M perchloric acid and was then hydrolyzed in 6 N HCl at 105° under vacuum for 22 hr. After removal of the hydrochloric acid, the residue was dissolved in H_2O, an aliquot was counted in a scintillation spectrometer, and an appropriate aliquot was used to separate hypusine and deoxyhypusine from other radioactive peaks.

The cell supernatant fraction was placed in an ice bath, and KOH, which precipitates perchloric acid as potassium perchlorate, was added to a slightly alkaline pH. The supernatant fraction, separated by centrifugation, was used to determine the concentration of the polyamines by HPLC and the distribution of counts. The counts were determined after separation of the polyamines by ion-exchange column chromatography and collection of fractions.

Separation and quantitation of polyamines by high-pressure liquid chromatography. The procedure used is a modification of the method of Seiler and Knodgen [10]. A Beckman high-pressure liquid chromatograph (model 334) was used with an Ultrasphere-IP column (Beckman) run at room temperature, and two buffers. Buffer A was 0.1 M sodium acetate containing 10 mM octane sulfonate at pH 4.5, and buffer B was a mixture of 0.2 M sodium acetate and acetonitrile in the proportion 3:2 (v/v) containing 10 mM octane sulfonate at pH 4.5. The gradient started at a flow rate of 1.0 ml/min with 50% buffer A and 50% buffer B. Buffer B was increased from 50% to 100% from 0 to 20 min. Polyamines were detected after post-column derivatization with the *o*-phthalaldehyde reagent delivered at a flow rate of 0.67 ml/min. A delay coil (10 ft long Teflon tubing, 0.3 mm i.d. × 1.5 mm o.d.) inserted between mixing T and detector improved both the sensitivity and reproducibility of the reaction. The resulting fluorescence was measured in a Gilson Spectra/Glo fluorometer equipped with a microflow cell and a Gilson filter with excitation 7-60X, emission 3-73M designed for *o*-phthalaldehyde. 1,7-Diaminoheptane was used as an internal standard.

Separation of hypusine, deoxyhypusine and polyamines by ion-exchange chromatography was accomplished in a short column (4 × 0.9 cm) of the Beckman 120B amino acid analyzer with the following buffers [11]: Buffer A contained 0.1 M sodium citrate and 0.3 M NaCl, pH 6.0; buffer B contained 0.1 M sodium citrate and 0.9 M NaCl, pH 5.7; and buffer C contained 0.1 M sodium citrate and 2.0 M NaCl, pH 5.2. Buffer A was run for 30 min, followed by Buffer B for 42 min and buffer C for a total of about 5 hr. The polyamines, putrescine, spermidine and spermine, were identified by comparison with authentic samples. Hypusine and deoxyhypusine were identified by the methods described below.

The peak tentatively identified as hypusine was desalted through a column (5 × 0.5 cm) of AG 50W-X8, a cation exchange resin in H^+ form. After washing the column extensively with water, hypusine was eluted with 4 M NH_4OH. The NH_4OH in the eluate was eliminated by lyophilization, and the residue containing hypusine was oxidized with periodate and permanganate. Periodate cleaves at the α-amino alcohol portion of hypusine producing β-aminopropionaldehyde, formaldehyde and lysine. Both β-aminopropionaldehyde and formaldehyde were oxidized further by the permanganate to β-alanine and formic acid [1, 3], and both were labeled when the cells were incubated previously with [1,4-^{14}C] putrescine. These oxidation products were identified by column, paper and thin-layer chromatography and comparison with the corresponding standards. The long column of the amino acid analyzer Beckman 120B was used to separate β-alanine and lysine; β-alanine was identified in the collected fractions by radioactive counting. Formic acid was not retained by the column, and counts appeared in the first fractions. For thin-layer chromatography, the following system was used: chloroform–methanol–14% aqueous NH_4OH solution (2:2:1, by vol.) and silica gel IB2 Baker plates [1]. Ascending paper chromatography was run on Whatman 1 paper with isopropanol–formic acid–water (4:1:1, by vol.). A procedure was developed for the rapid identification of labeled hypusine. It was based on the precipitation of labeled formaldehyde, a product of periodate oxidation of hypusine, with dimedone [12]. Formaldehyde was determined by radioactivity counting of the dimedone precipitate collected by filtration in a 0.45 μm Millipore filter in a scintillation spec-

trometer. To the filter placed in a vial was added 0.5 ml of 0.1 N HCl and 10 ml of a toluene-based scintillation fluid.

Identification of deoxyhypusine. The chromatographic properties of the peak tentatively identified as deoxyhypusine were compared to those of an authentic sample [2] provided by Drs. Folk and Cooper from the National Institutes of Health. This sample was derivatized as the trifluoroacetyl methyl ester and the mass spectrum was determined. The following peaks and the relative intensities (in parentheses) M^+, *m/e* 519 (43%); M^+-32, *m/e* 487 (55%); M^+-59, *m/e* 460 (45%); M^+-69, *m/e* 450 (54%); M^+-97, *m/e* 422 (80%); M^+-113, *m/e* 406 (20%); and M^+-154, *m/e* 365 (100%) were consistent with the structure of trifluoroacetyl deoxyhypusine methyl ester. An additional standard material ε-(4-benzamidobutyl)-lysine hydantoin hydrobromide was also prepared in our laboratory. It was obtained by the reductive amination [13] of 4-benzamidobutyraldehyde with excess lysine hydantoin in the presence of sodium cyanoborohydride. The crude material examined by mass spectrometry showed the following peaks: M^+, *m/e* 346 (87%); *m/e* 241 (50%); *m/e* 205 (95%); *m/e* 198 (85%) and *m/e* 184 (100%) which indicated the presence of ε-(4-benzamido-butyl)-lysine hydantoin in the mixture. This mixture was hydrolyzed with 6 N HCl under vacuum at 105° for 22 hr. After removal of the HCl, an aliquot of the solution in H_2O was chromatographed on the short column of the amino acid analyzer as described for the separation of deoxyhypusine, hypusine and polyamines. The chromatograph showed two major ninhydrin positive peaks, one which was identified as lysine and the other which eluted in the position of standard deoxyhypusine. This latter peak was isolated, desalted, and chromatographed in the same column with labeled deoxyhypusine isolated from a cell protein hydrolysate. The ninhydrin positive peak of racemic deoxyhypusine and the counts were in the same position.

Separation of the hypusine-containing protein. The hypusine-containing protein was identified by two-dimensional gel electrophoresis following the procedure of O'Farrell [14]. The methods of Lowry *et al.* [15] and of Fiszer-Szafarz *et al.*[16] were used for protein and DNA determination respectively.

RESULTS

Logarithmically growing CHO cells incubated in the presence of [1,4-^{14}C]putrescine synthesize a labeled-hypusine-containing protein. This unusual amino acid was isolated from the cell protein hydrolysate by ion-exchange chromatography, as described under Materials and Methods, and is shown in Fig. 1. In the chromatogram (Fig. 1), between 80 and 90% of the counts were present in the hypusine peak (tubes 20–25) and the remaining counts were present in the elution positions of putrescine, spermidine and spermine. Early eluting peaks which have not been identified, contained about 5% of the counts. These small peaks were always present in spite of the extensive washing of the cell protein after precipitation with perchloric acid. The peak in tubes 20–25 was identified as hypusine after desalting and periodate

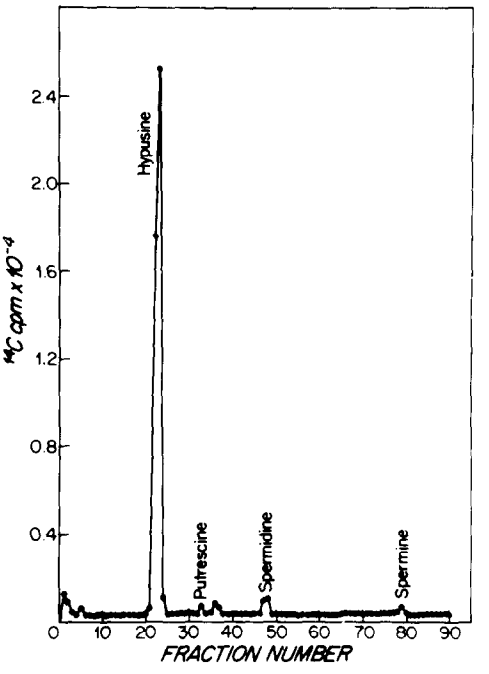

Fig. 1. Ion-exchange chromatography of a hydrolysate of the protein fraction from CHO cells incubated with [1,4-^{14}C]putrescine. Cells were seeded and cultured as described in Materials and Methods. Twenty-four hours after seeding, the medium was changed to fresh medium, and 2 hr later [1,4-^{14}C]putrescine (sp. act. 102 mCi/mmole) was added at a concentration of 0.25 μCi/ml and the cells were incubated for 48 hr. The acid hydrolysate of the cell protein precipitated with perchloric acid was chromatographed, and fractions of 3.3 ml were collected every 3.5 min.

oxidation (Table 1). The identification was also accomplished by periodate permanganate oxidation and separation of the oxidation products by ion-exchange column, paper, and thin-layer chromatography. The R_f values of lysine and β-alanine were 0.14 and 0.52 (paper), and 0.15 and 0.49 (thin-layer). Counts were present only in the position of β-alanine.

Incubation with hydralazine added to the culture medium at 0.2 mM for 48 hr showed a 40–80% inhibition of hypusine hydroxylation in several experiments with only slight inhibition of deoxyhypusyl residue synthesis. Consequently, there was accumulation of deoxyhypusyl residues, as shown in Fig. 2. A new peak in tubes 25–30, which was identified as deoxyhypusine, appeared in the chromatogram of the cell protein hydrolysate. In this particular experiment, 0.2 mM hydralazine caused almost 80% inhibition of hypusine hydroxylation. In addition, hydralazine produced a 40% decrease in total protein synthesis and about an 80% decrease in DNA synthesis compared to a control cultured without the drug and incubated for the same period of time. When different doses of hydralazine were studied to determine a dose–response effect on hypusine hydroxylation, it was found that 0.05 mM had a negligible effect on hypusine hydroxylation and 0.1 mM caused 13–16% inhibition of hypusine hydroxylation. The highest dose, 0.3 mM, resulted

Table 1. Identification of labeled hypusine isolated from the protein hydrolysate of cells incubated with [1,4-^{14}C]putrescine*

Periodate oxidation	Dimedone supernatant	Dimedone adduct precipitate
	Radioactivity (cpm)	
Hypusine + iodate + dimedone (control)	8912	40
Hypusine + periodate + dimedone	4570	4120

* Ion exchange chromatography of an acid hydrolysate of the perchloric acid-insoluble protein fraction of the cell was carried out. The peak eluted at the position of hypusine was desalted through a column of AG 50W-X8. The fraction eluted from the desalting column with 4 M NH_4OH was lyophilized and oxidized with periodate. The formaldehyde produced was precipitated with dimedone, and the radioactivity in the precipitate and supernatant fraction was determined. Iodate used as control did not cleave the hypusine molecule, and formaldehyde was not produced. Radioactivity in the hypusine peak after desalting was 9540 cpm.

in almost complete inhibition of protein and DNA syntheses with clear evidence of toxicity as many cells peeled off the surface of the culture flask. In view of these results, 0.2 mM hydralazine was used for all the experiments described, although some variation on its effect on hypusine hydroxylation and on cell toxicity was observed in some experiments. Figure 3 summarizes the results obtained when logarithmically growing CHO cells were incubated with hydralazine and [^{14}C]putrescine. After 24 hr in culture, hydralazine caused extensive inhibition of the hydroxylation reaction, and more than 80% of the protein-bound counts appeared in deoxyhypusine In the control culture without hydralazine, only hypusine was present. When the culture was continued for another 24 hr without adding additional drug, the cells were able to synthesize new hypusine and to hydroxylate some of the accumulated deoxyhypusine, reducing the apparent inhibition of hypusine hydroxylation to about 40%. When the medium was changed at 24 hr to fresh medium containing neither hydralazine nor [^{14}C]putrescine, a marked reversion of the drug effect was observed. All but 3% of the accumulated deoxyhypusine was converted to hypusine, and new synthesis of hypusine occurred. In the control cells without drug and continuing culture for 48 hr, there was a significant increase in the amount of hypusine synthesized per flask compared to 24 hr. The difference was more marked when the medium was changed at 24 hr and the cells were incubated for an additional 24 hr period.

The effects of hydralazine on protein, DNA, hypusine and deoxyhypusine synthesis are presented in Table 2. Both protein and DNA syntheses were affected by the drug but to a different degree. After

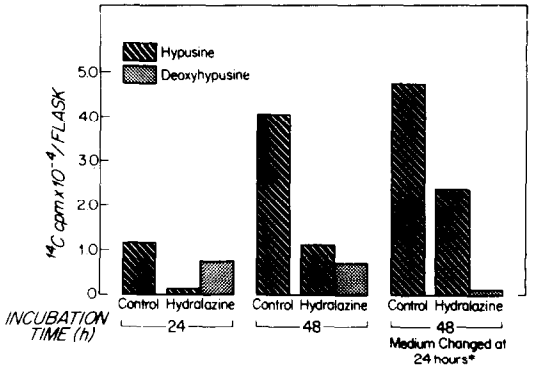

Fig. 3. Effect of hydralazine on deoxyhypusine and hypusine synthesis in CHO cells cultured in the presence of [1,4-^{14}C]putrescine. Cells were seeded and cultured as described in Materials and Methods and in the legends of Figs. 1 and 2. After the addition of [1,4-^{14}C]putrescine the cells were incubated for 24 or 48 hr without changing the medium. (*) The cells were incubated for 48 hr with a medium changed at 24 hr to fresh medium that did not contain hydralazine or [^{14}C]putrescine.

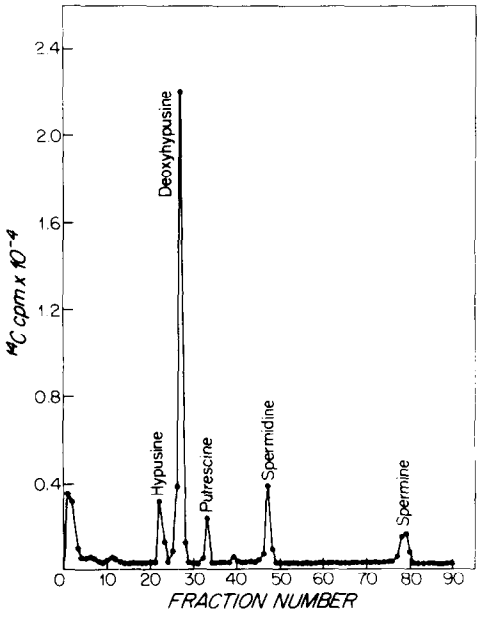

Fig. 2. Ion-exchange chromatography of a hydrolysate of the protein fraction from CHO cells incubated with 0.2 mM hydralazine and [1,4-^{14}C]putrescine. Cells were seeded and cultured as described in Materials and Methods. Twenty-four hours after seeding, the medium was changed to fresh medium containing 0.2 mM hydralazine and 2 hr later [1,4-^{14}C]putrescine was added. Procedure and conditions are the same as in Fig. 1.

Table 2. Effect of hydralazine on protein, DNA, hypusine and deoxyhypusine syntheses*

Treatment	Incubation time (hr)	DNA (μg)	Protein (mg)	Hypusine (cpm/mg protein)	Deoxyhypusine (cpm/mg protein)
Control	24	176.1	9.0	5050	Not present
Plus hydrazine	24	76.8	8.3	505	3489
Control	48	729.0	21.7	7462	Not present
Plus hydrazine	48	80.4	11.1	3983	2490
Control	48†	608.2	22.0	8693	Not present
Plus hydrazine	48‡	128.1	14.3	6547	206

* Chinese hamster ovary cells were seeded at 2×10^6 cell density per T150 flask and were incubated for 24 hr. The medium was then changed to fresh medium with or without 0.2 mM hydralazine. After 2 hr of incubation, 0.25 μCi/ml of [^{14}C]putrescine was added. The cells were further incubated for 24 hr or 48 hr and were then processed as described under Materials and Methods.

† The medium was changed at 24 hr to fresh medium without [^{14}C]putrescine.

‡ The medium was changed at 24 hr to fresh medium that did not contain [^{14}C]putrescine or hydralazine.

24 hr, a slight decrease in protein synthesis compared to the control was observed. In contrast, DNA synthesis decreased about 56% during the same period of time. Continuous culture in the presence of the drug for 48 hr resulted in almost 50% decrease in protein synthesis and about a 90% decrease in DNA synthesis. The experiment presented in Table 2 is representative of three other experiments in which very similar effects of 0.2 mM hydralazine on protein and DNA syntheses were observed at 24 and 48 hr. Removal of the drug by changing the medium to fresh medium resulted in some recovery of the cells as determined by an increase in the synthesis of protein and DNA compared to the cells cultured continuously in the presence of the drug for 48 hr. The medium change produced a burst in DNA synthesis which was detected by an increase in the number of labeled nuclei when cells were incubated with [^{3}H]thymidine. This increase is not reflected in the numbers in Table 2, probably due to the loss of 20–30% of the cells that were in mitosis and loosely attached to the surface of the flask, when the conditioned medium was poured off and replaced with fresh medium. Fewer cells were lost from the hydralazine-treated cultures during the change of medium as fewer cells were in mitosis compared to the control. It is clear from the examination of Fig. 3 that the amount of hypusine plus deoxyhypusine synthesized per flask by the treated cells was less than the amount of hypusine synthesized by the control cells. This difference is less marked when the results are expressed per mg of cell protein, as in Table 2. The cell supernatant fraction was examined to determine the concentration of polyamines and the metabolic fate of the putrescine incorporated by the cells (Table 3).

Most of the counts present in the cell supernatant fraction were distributed among putrescine, spermidine and spermine with less than 3% of the counts in as yet unidentified peaks. The distribution of counts in the polyamines was very similar whether or not the cell supernatant fraction was hydrolyzed, indicating that the polyamines were present mainly as free

Table 3. Distribution of radioactivity in the polyamines of the perchloric acid supernatant fraction of CHO cells incubated with [^{14}C]putrescine in the presence or absence of hydralazine*

Treatment	Incubation time (hr)	Putrescine (%)	Spermidine (%)	Spermine (%)
Control	24	23.9 (13.6)	59.3 (8.6)	15.6 (4.9)
Hydralazine	24	54.8 (15.3)	38.2 (8.6)	4.9 (2.3)
Control	48	1.8 (0.48)	54.2 (4.1)	43.0 (6.6)
Hydralazine	48	29.8 (12.7)	55.6 (11.8)	12.3 (7.8)
Control	48†	2.1 (0.54)	55.6 (4.1)	41.3 (5.1)
Hydralazine	48‡	12.0 (4.4)	66.0 (7.0)	20.4 (8.6)

* Cells were seeded and incubated as indicated in the legend to Table 2. After the incubation period, the cells were washed, homogenized and precipitated with perchloric acid. The supernatant fractions separated by centrifugation were analyzed by ion-exchange chromatography to determine the distribution of radioactivity and by HPLC to determine the concentrations of polyamines as described under Materials and Methods. Numbers in parentheses represent specific radioactivities in cpm $\times 10^{-4}$ per nmole.

† The medium was changed at 24 hr to fresh medium without [^{14}C]putrescine.

‡ The medium was changed at 24 hr to fresh medium that did not contain [^{14}C]putrescine or hydralazine.

compounds. Putrescine, incorporated into the cells, was metabolized to spermidine which, in turn, was converted to spermine. The data in Table 3 show that the treated cells metabolized putrescine more slowly than the control cells and that, after 24 hr of incubation, 55% of the counts were still present as putrescine, compared to 24% in the control. Moreover, at this time point, the putrescine concentration was almost four times higher in the hydralazine-treated cells than in the control, accounting for the similar specific radioactivities. Spermidine and spermine, in contrast, were present in similar concentrations. The percentage of counts in spermidine did not vary significantly, and the difference between controls and treated cells was slight. This polyamine remained essentially constant after reaching a certain level within the cell, the excess being rapidly converted into spermine. Consequently, the percentage of counts in spermine was higher in the control cells than in the treated cells as more putrescine was metabolized in the control cultures. From 24 to 48 hr, a marked decrease in the concentration of polyamines in the hydralazine-treated cells was observed, suggesting an inhibitory effect of the drug on polyamine synthesis. The medium change at 24 hr, to fresh medium containing no hydralazine, stimulated cell division and improved the utilization of putrescine, reducing its percentage at 48 hr from 30 to 12%. Examination of the hydrolyzed medium (not shown) from the experiments above showed a very small number of counts in spermine, suggesting that the cells had excreted mainly spermidine, as was also observed by Melvin and Keir [17]. Hydralazine did not affect the uptake of [^{14}C]putrescine, and both the control and the treated cells incorporated the same number of counts. This incorporation accounted for 70–80% of the counts in the medium at 24 hr, and more than 99% of the counts were present in the cell supernatant fraction with less than 1% in the cell protein. In the cell protein, all the counts were present in a single protein that contained the amino acid hypusine. This protein, isolated by two-dimensional gel electrophoresis, appeared to have a molecular weight of approximately 18,000 and a slightly acidic pI. Figure 4A shows the gel stained with Coomassie Blue and the arrow points to the position of the hypusine-containing protein. Figure 4B shows the radioautography demonstrating essentially a single radioactive spot. No other spots appeared after prolonged exposure of the film (4 weeks).

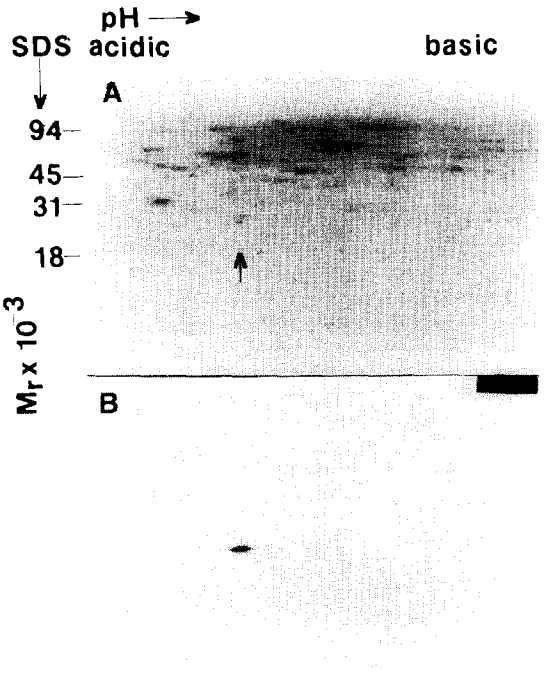

Fig. 4. Two-dimensional polyacrylamide gel electrophoresis of [^{14}C]putrescine-labeled proteins from CHO cells. The cells were cultured as in Fig. 1. After the cell homogenization, the protein was precipitated with perchloric acid to 0.4 M final concentration. The cell protein was dissolved and the electrophoresis performed as described by O'Farrell [14]. (A) Pattern of protein staining with Coomassie Blue. (B) Fluorogram prepared with the same gel using EN^{3}HANCE (New England Nuclear) and exposed to Kodak X-Omat AR film for 4 weeks. The arrow points out the position of the labeled-hypusine-containing protein.

DISCUSSION

Chinese hamster ovary cells, in the logarithmic phase of growth, synthesize a protein(s) that contains the amino acid hypusine. A step in the post-translational synthesis of this unusual amino acid, which involves a hydroxylation reaction, was strongly inhibited by the addition to the culture medium of hydralazine (1-hydrazinophthalazine, apresoline). Hydralazine, introduced as an effective hypotensive agent in 1951, has been shown by us [8] and others [18] to inhibit collagen prolyl hydroxylation. This reaction, carried out by the enzyme prolyl hydroxylase, a mixed function oxidase, requires as essential cofactors α-ketoglutarate, O_2 and iron, with ascorbate as the most effective reducing agent and mediator. Reduction of the content of any of these factors by hydralazine may result in the inhibition of the prolyl hydroxylase. Acting as a metal chelator, hydralazine may remove iron and as a carbonyl reagent, may react with α-ketoglutarate [19] causing a depletion of this essential cofactor at the site of collagen hydroxylation. It is more likely, however, that hydralazine as a strong reducing agent depletes electron accepting intermediates involved in a variety of hydroxylative reactions. Chronic administration of hydralazine to patients can lead to a syndrome resembling disseminated lupus erythematosus, which probably has an immunologic basis [20].

In this paper, we have shown that hydralazine caused extensive inhibition of hypusine hydroxylation, suggesting that the enzyme, deoxyhypusyl hydroxylase(s), may be an oxygenase similar to prolyl hydroxylase. Park *et al.* [2] have found recently that agents such as α,α-dipyridyl, picolinic acid and desferal, which are metal chelators, inhibit hypusine hydroxylation. Several possible cofactors in the reaction (α-ketoglutarate, $FeSO_4$ and ascorbate) failed to enhance the deoxyhypusine to hypusine conversion in their cell-free system.

In addition to its inhibitory effect on hypusine hydroxylation, hydralazine had an effect on total protein synthesis. At 24 hr the effect was slight, but prolonging the culture for 48 hr in the presence of the drug caused a further reduction on total protein synthesis to 40–50% of the value in the control culture. The protein that contained hypusine did not appear to be affected to the same extent as total protein synthesis. As shown in Table 2, the level of this protein was reduced by only 20% compared to the control at 48 hr. In contrast, hydralazine had a marked effect on DNA synthesis and cell multiplication. Such an effect may be secondary to the decrease in polyamine concentration and utilization (Table 3) observed in the treated cells. In several studies [21–23] a close relationship has been shown between increases in polyamine biosynthesis as well as polyamine concentration and the rate of cell proliferation. Moreover, a decrease in spermidine biosynthesis from putrescine may have effects on cell division [24] and on the synthesis of hypusine, since spermidine is the precursor of this amino acid [3]. The dramatic inhibitory effect of hydralazine on the post-translational synthesis of hypusine coupled with the strong decline in the synthesis of DNA could be of importance in cell cycle regulation. Such a role could require complete hydroxylation of deoxyhypusine to hypusine. It is noteworthy that, within 24 hr after removal of the drug from the medium, almost complete reversal of the effect on hypusine hydroxylation and, in part, the effect on total protein and DNA syntheses was seen.

Hydralazine appears to be a useful drug for the study of the hydroxylation reaction leading to the synthesis of hypusine. The deoxyhypusyl-containing protein that accumulates in the presence of hydralazine may be used to prepare the natural substrate for the hydroxylase enzyme, facilitating future investigations on the properties and the co-factor requirements of the enzyme(s) involved. Moreover, the hydroxylation reaction may be an essential step in the biosynthetic processing of the hypusine-containing protein for its functional activity. Examination of the cell metabolic activity, in particular DNA synthesis and cell division, in relation to various degrees of hypusine hydroxylation inhibition, may contribute to further understanding of cell cycle regulation and cellular growth. Since recent evidence now suggests that the hypusine-containing protein may be a translation initiation factor in protein synthesis [25], a pattern begins to emerge relating the familiar growth-stimulating properties of the polyamines to a direct effect on the initiation of protein synthesis. It may be appropriate to view this stepwise post-translational reaction, involving spermidine and a lysyl residue of a specific initiation factor, as a new type of polyamine-dependent second messenger reaction. The inhibition by hydralazine of the hydroxylative component of this reaction leads one to question whether side reactions in the clinical use of this hypotensive agent might be related to this inhibition. The recommended clinical doses of hydralazine may be as high as 400 mg/day. For a 70 kg adult, this is approximately a concentration of 0.03 mM. Therefore, if an individual patient had some unrelated problem that decreased the metabolism of this drug, the concentrations which were studied here and found to inhibit deoxyhypusine hydroxylation might well accumulate.

Acknowledgements—We are grateful to Dr. Fred Rosen for his helpful advice and discussions; to Drs. Myung H. Park, Herbert L. Cooper and J. E. Folk, from the National Institutes of Health, Bethesda, MD, for making available to us the results of some of their findings before publication and for the sample of deoxyhypusine; to Dr. John Walters for the two-dimensional gel; and to Kathleen Pearson and Jennifer Brill for expert technical assistance.

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