MeSCap (^{14}C:^{15}N = 1.0:0.55) this deviation from a 1:1 relationship of ^{14}C:^{15}N in product progoitrin may reflect transamination or oxidative deamination of DL-NH_2MeSCap with subsequent loss of ^{15}N label to the amino acid pool (Table III). Matsuo and Yamazaki (1966) observed similar losses of ^{15}N when studying DL-[^{14}C,^{15}N]homomethionine incorporation into sinigrin. They ascribed these losses to utilization of the unnatural D form of the amino acid after its deamination to the corresponding keto acid. Similar circumstances may obtain with D-NH_2MeSCap.

The efficiency of utilization of NH_2MeSCap in progoitrin formation (Lee and Serif, 1968), the retention of the carbon–nitrogen bond of NH_2MeSCap and the specific incorporation of the C-2 of NH_2MeSCap into the C-1 position of progoitrin all support the precursor role for NH_2MeSCap outlined in Figure 2 and suggest the existence of NH_2MeSCap as a normal plant metabolite.

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Solid-Phase Synthesis of [5-Glutamine]-α-melanotropin*

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ABSTRACT: The tridecapeptide [5-glutamine]-α-melanotropin, a new analog of the hormone, has been synthesized by the solid-phase method.

The fully protected tridecapeptide was cleaved from the resin by methanolysis in dimethylformamide–methanol–triethylamine. Ammonolysis of the methyl ester was effected in dimethylformamide–ethylene glycol–water; the product was then reduced with sodium in liquid ammonia and purified chromatographically to give the highly purified and biologically active tridecapeptide.

Fractionation of the pituitary extracts (Lerner and Lee, 1962; Li, 1959) from several animal species has led to the isolation of a tridecapeptide designated as α-melanotropin (α-MSH).[1] The structure has been shown to be identical in the species investigated, namely *N*-acetylseryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalinamide. In addition to its ability to darken the skin of certain amphibians, α-MSH has been shown to possess a variety of other biological activities (Bowers *et al.*, 1964; Krivoy and Guillamin, 1961). Synthesis of the natural hormone by classical techniques has been reported by two groups (Guttmann and Boissonnas, 1959; Schwyzer *et al.*, 1963), and the synthesis of a variety of tridecapeptide and smaller peptide analogs has also been described (Schröder and Lübke, 1966). Recently we reported the synthesis by the solid-phase method of a heptapeptide, methionylglutamylhistidylphenylalanylarginyltryptophylglycine (Blake and Li, 1968), an active core of α-MSH. As an extension of that synthesis, we have synthesized the peptide [5-glutamine]-α-MSH, a new analog of α-MSH, which is reported here.

The synthesis of [5-glutamine]-α-MSH followed the Merrifield procedure (Merrifield, 1964) with the modifications previously reported (Blake and Li, 1968). Deblocking of the Boc group was accomplished with 3.6 N HCl in dioxane, using β-mercaptoethanol to protect tryptophan from acid-

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[1] Abbreviations used are: MSH, melanocyte-stimulating hormone, melanotropin; Boc, *t*-butyloxycarbonyl. All amino acids occurring in the peptides mentioned in this paper are of the L configuration with the exception of glycine.

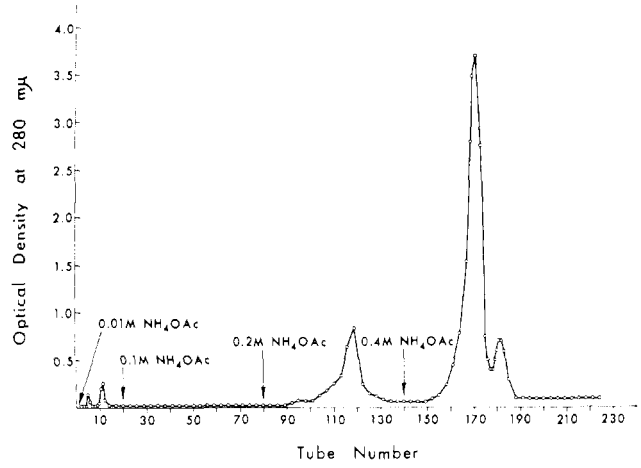

FIGURE 1: CM-cellulose chromatography (column, 1.4 × 55 cm) of crude [5-glutamine]-α-MSH. The initial buffer was 0.01 M ammonium acetate, pH 4.5. After 20 tubes (10 ml/tube), a gradient with respect to pH and salt concentration was started by introducing 0.1 M ammonium acetate buffer of pH 6.7 through a 500-ml mixing flask containing the starting buffer. Thereafter the volume per tube was 4 ml. Later the gradient was increased by substituting higher concentration buffers at the indicated positions in the figure.

catalyzed oxidation (Marshall, 1968). Coupling was achieved with 4 equiv of Boc-amino acid and *N,N'*-dicyclohexylcarbodiimide (Sheehan and Hess, 1955). Glutamine was coupled to the peptide-resin by means of its Boc ONp derivative, and deblocking of the N-terminal glutamine peptide was effected with 50% trifluoroacetic acid in methylene chloride to prevent cyclization (Takashima *et al.*, 1968) of the glutamine residue. The amino acid side chains were protected as follows: serine and tyrosine, *O*-benzyl; lysine and arginine, tosyl; histidine, Im-benzyl.

Preliminary experiments with Boc-Val-resin indicated that the usual ammonolysis procedure of ammonia in methanol (as employed in the synthesis of deaminooxytocin) (Takashima *et al.*, 1968) and oxytocin (Manning, 1968) gave mainly the methyl ester and very little amide, as reported elsewhere (Bodansky and Sheehan, 1966) for a carboxyl-terminal valine peptide. A higher yield of Boc-ValMe was obtained by the method of Beyermann *et al.* (1968), involving treatment of the resin with dimethylformamide–methanol–triethylamine, and it was this procedure that was employed to cleave the protected tridecapeptide from the resin to give a 81% yield of methyl ester. Treatment of a dimethylformamide–ethylene glycol–water solution (Gordon *et al.*, 1949) of the tridecapeptide methyl ester with ammonia, followed by reduction with sodium in liquid ammonia (du Vigneaud and Behren, 1937) and purification by carboxymethylcellulose chromatography (Peterson and Sober, 1956) (Figure 1) gave [5-glutamine]-α-MSH.

MSH assay by the *in vivo* procedure (Hogben and Slome, 1931) showed that a dose of 0.008 μg of [5-glutamine]-α-MSH produced a change in melanophore index in hypophysectomized *R. pipiens* from 1^+ to 3^+ within 1 hr. MSH assay by the *in vitro* procedure (Shizume *et al.*, 1954) showed an activity of 1×10^9 units/g for the synthetic analog of α-MSH as compared with ovine adrenocorticotropin of 2×10^7 units/g. The natural α-MSH is known to contain 1×10^{10} units/g as assayed (Lerner and Lee, 1962; Li, 1959) by the *in vitro* procedure. It is of interest that a replacement of glutamic acid by glutamine in the α-MSH molecule causes a marked decrease of the melanocyte-stimulating activity.

Experimental Section

Boc-Val-Resin. To a solution of 1.65 g (7.6 mmoles) of Boc-valine and 1.0 ml of triethylamine in 22 ml of anhydrous ethanol was added 5 g of Bio-beads[2] S-X-2, 200–400 mesh, chloromethylated at 0.72 mequiv/g. The mixture was stirred under reflux for 20 hr and filtered, and the resin was exhaustively washed with ethanol, dilute acetic acid, water, and methanol. A sample of the dried Boc-valyl-resin was hydrolyzed in 1:1 dioxane–constant-boiling HCl, and amino acid analysis (Spackman *et al.*, 1958) showed the valine content to be 0.32 mmol/g.

N-Acetyl-O-benzylseryl-O-benzyltyrosyl-O-benzylserylmethionylglutaminyl-Im-benzylhistidylphenylalanyl-N^G-tosylarginyltryptophylglycyl-N^ϵ-tosyllysylprolylvalyl-resin. A portion of the Boc-Val-resin (3.9 g, 1.25 mmoles of valine) was treated by the following steps: (1) three washings with 20-ml portions of dioxane; (2) cleavage of the Boc group by addition of 18 ml of 5.6 N HCl in dioxane (diluted by dioxane held up by resin in step 1 to yield an effective concentration of 3.6 N HCl) and shaking for 30 min; (3) washing with two 20-ml portions of dioxane, three 20-ml portions of ethanol, and three 20-ml portions of chloroform; (4) neutralization of the hydrochloride with 20-ml of chloroform and 2-ml of triethylamine for 10 min; (5) washing with three 20-ml portions of chloroform and three 20-ml portions of methylene chloride; (6) addition of 5.0 mmoles of Boc-amino acid in 12 ml of methylene chloride and shaking for 10 min; (7) addition of 5.0 mmoles of *N,N'*-dicyclohexylcarbodiimide in 4 ml of methylene chloride and shaking for 3 hr; (8) three washings with 20-ml portions of dimethylformamide; (9) acetylation by addition of 20-ml of dimethylformamide, 1 ml of acetic anhydride, and 0.4 ml of *N*-methylmorpholine, and shaking for 20 min; (10) washing with one 20-ml portion of ethanol, three 20-ml portions of acetic acid, and three 20-ml portions of ethanol.

After the introduction of tryptophan into the peptide resin, 0.2 ml of β-mercaptoethanol was incorporated into step 2. Coupling of glutamine was achieved with 6.3 mmoles of Boc-glutamine *p*-nitrophenyl ester in dimethylformamide and a reaction time of 22 hr. Deblocking of the N-terminal glutamine peptide was accomplished by shaking the resin with 50% trifluoroacetic acid in methylene chloride for 15 min. For this cycle methylene chloride was substituted for dioxane in steps 1 and 3, and dimethylformamide was substituted for methylene chloride in step 5.

After the final coupling cycle, the peptide was deblocked according to steps 1, 2, and 3, washed with two 25-ml portions of dimethylformamide, and acetylated by addition of 25 ml of dimethylformamide, 2 ml of acetic anhydride, and 0.45 ml of triethylamine, and shaking for 75 min. The peptide-resin was then washed with two 25-ml portions of dimethylformamide and four 30-ml portions of ethanol, and dried.

[2] Commercially available from Bio-Rad Laboratories, Richmond, Calif.

N-Acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalinamide. ([*5-Glutamine*]-α-*MSH*). To a portion of the above-protected tridecapeptide-resin (4.9 g, 0.84 mmole) was added 50 ml of dimethylformamide, 50 ml of methanol, and 22 ml of triethylamine. The mixture was stirred at 42° for 22 hr, filtered, the resin was washed with dimethylformamide, and the combined filtrate was evaporated. The resin was retreated by the same procedure for another 22 hr, filtered, and the filtrate was evaporated to a residue which was combined with the residue of the first filtrate. Spectral analysis at 280 mμ indicated that 0.68 mmole (81%) of peptide had been cleaved from the resin.

The above methyl ester was dissolved in 110 ml of dimethylformamide, 79 ml of ethylene glycol, and 51 ml of water. The solution was saturated with ammonia at 0° and stored in a pressure bottle at room temperature. After 20 hr the solution was resaturated with ammonia at 0°, and stored for 2 more days in a pressure bottle at room temperature. At this time the mixture was evaporated to *ca.* 80 ml, and the peptide was precipitated by the addition of water. After cooling at 0°, the mixture was centrifuged and the precipitate was washed with water and dissolved in dimethylformamide. Spectral analysis indicated the presence of 0.68 mmole of peptide.

A portion of the above dimethylformamide solution, containing 0.044 mmole of peptide, was evaporated and thoroughly dried. The peptide was dissolved in 120 ml of liquid ammonia, freshly distilled from sodium. Small pieces of sodium were added until the blue color persisted[3] for 20 min. The solution was evaporated to dryness, and the residue was desalted on IRC-50 resin, and eluted with pyridine–acetic acid–water (30:4:66). The lyophilized crude tridecapeptide was purified by carboxymethylcellulose chromatography (see Figure 1) using ammonium acetate gradient elution to yield, after three lyophilizations, 26 mg of [5-glutamine]-α-MSH (peptide content 77%, 0.012 mmole, 22% yield based on starting Boc-Val-resin); $[\alpha]_D$ −58° (*c* 0.38, 10% acetic acid) [lit. (Schwyzer *et al.*, 1963) for α-MSH, $[\alpha]_D$ −58.5° (*c* 0.38, 10% acetic acid); $[\alpha]_D$ −56° (*c* 0.5, 10% acetic acid)].

Paper electrophoresis in pyridine acetate buffer (pH 3.7, 400 V, 4 hr) showed one ninhydrin-positive, Pauly-positive, and Sakaguchi-positive spot at R_F 0.60 (with respect to lysine). Paper electrophoresis in collidine acetate buffer (pH 6.9, 400 V, 4 hr) showed one ninhydrin-positive, Pauly-positive spot at R_F 0.50. Thin-layer chromatography on silica gel in the system 1-butanol–pyridine–acetic acid–water (15:10:3:12) showed one ninhydrin-positive, chlorine-positive spot at R_F 0.5. Amino acid analysis (Spackman *et al.*, 1958) of an acid hydrolysate gave: $Ser_{1.73}Tyr_{0.99}Met_{0.99}Glu_{1.00}His_{0.98}Phe_{1.00}Arg_{1.03}Gly_{1.02}Lys_{1.01}Pro_{0.05}Val_{0.97}$. Measurement of the ultraviolet spectrum (Beaven and Holiday, 1952) in 0.1 N sodium hydroxide solution showed Tyr/Trp = 1.02. Microbiological assay[4] of an acid hydrolysate for L-amino acids gave $Phe_{1.0}Tyr_{0.9}Arg_{1.0}His_{0.9}$. These amino acids were assayed microbiologically because it was believed that they would be particularly susceptible to racemization, if any was to occur, by the basic conditions (Geschwind and Li, 1964) employed in the methanolysis and ammonolysis reactions.

For determination of the carboxyl-terminal amide content, 2.7 mg of the tridecapeptide was dissolved in 1 ml of pH 8.5 ammonium acetate buffer, 0.09 mg of chymotrypsin was added, and the solution was stored at 37° for 26 hr. After acidification and lyophilization, the sample was subjected to preparative paper electrophoresis (pH 6.9, 400 V, 4 hr). The band corresponding to the fastest moving basic peptide (Harris, 1959) was cut out and eluted with 0.1 N acetic acid. Acid hydrolysis of this peptide and amino acid analysis gave $Gly_{0.9}Lys_{1.0}Pro_{1.0}Val_{1.0}amideNH_{3_{1.0}}$.

[3] In response to an inquiry by a referee, we have observed no evidence of cleavage of the Lys–Pro bond during the reduction with sodium in liquid ammonia.

[4] Microbiological assay was performed by Shankman Laboratories, Los Angeles, Calif.

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Cell-Free Synthesis of Ferredoxin in Clostridial Extracts*

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ABSTRACT: Amino acids are incorporated into a specific protein, ferredoxin, in a cell-free system from *Clostridium pasteurianum*. Preparations most active in cell-free synthesis were prepared from osmotically shocked protoplasts. Evidence that ferredoxin was synthesized *in vitro* was provided by cochromatography of radioactive tryptic peptides with carrier peptides of ferredoxin. Comigration of radioactivity with carrier peptide was also demonstrated by paper electrophoresis. Only low levels of radioactivity were associated with the N-terminal tripeptide. Incorporation of amino acids into ferredoxin appears therefore, to be due largely to completion of preexisting peptide chains. The effects of variation in the growth condition on *in vitro* ferredoxin synthesis were also examined.

Studies, *in vitro*, of peptide synthesis have been most important for defining the biochemistry of protein synthesis. In order to relate the *in vitro* events to those occurring in the cell, however, it is necessary to study the synthesis of well-defined proteins. This is especially important for an understanding of possible controls at the level of translation or for elucidating the function of other cellular components such as membranes in protein synthesis.

There are now a number of reports of the *in vitro* synthesis of proteins. These studies include a variety of cellular proteins (Bishop *et al.*, 1960; von Ehrenstein and Lipmann, 1961; Bonner, 1965; Ganoza *et al.*, 1965; Nisman *et al.*, 1961; Lederman and Zubay, 1968) as well as those coded by viral RNAs (Nathans *et al.*, 1962; Nathans, 1965; Clark *et al.*, 1965; von Ravenswaay Classen *et al.*, 1967) or the T-4 phage genome (Salser *et al.*, 1967). The evidence provided has ranged from cochromatography of tryptic digests of the *in vitro* product with carrier peptides to increases of enzyme activity or of precipitate formed upon addition of a specific antibody. Aside from the generally recognized problem of intact cell (or protoplast) contamination (Tonomura and Rabinowitz, 1967), there are dangers of increased enzyme activity being due to activation (Rogers, 1965) or of fortuitous binding in immunological studies.

While these pitfalls have been more or less controlled, we felt they could be bypassed by studying the synthesis of a protein which can be readily isolated and characterized. For this reason, we have undertaken a study of the *in vitro* synthesis of a native bacterial protein, ferredoxin.[1] Many of the properties of Fd appeared ideal for the study of its *in vitro* synthesis. Since the initial characterization of Fd from *Clostridium pasteurianum* by Mortenson *et al.* (1962), the chemistry of this protein has been extensively documented. Fd is a low molecular weight protein which constitutes about 1% of the clostridial protein. Its amino acid composition is unusual in that leucine, arginine, methionine, histidine, and tryptophan are absent. The amino acid sequence of clostridial Fd has been determined (Tanaka *et al.*, 1964a). Because of its unique chemical properties, Fd can be readily isolated and purified by solvent fractionation and anion-exchange chromatography (Mortenson, 1964a). It seemed reasonable therefore, to expect that sufficient radioactive material could be obtained from an *in vitro* amino acid incorporation system to permit a definitive characterization of the products. In this communication, we report the development of a bacterial cell-free system from *Clostridium pasteurianum*, which can synthesize Fd.

Materials and Methods

Cultivation and Harvest. C. pasteurianum was grown at 30° according to the method of Carnahan *et al.* (1960) with sucrose and N_2 as the carbon and nitrogen sources, respectively (generation time 2–3 hr), unless stated otherwise. To prepare cell-free extracts for amino acid incorporation, cultures were harvested during exponential growth (3–5 × 10^8 cells/ml).

Escherichia coli (strain KB) was grown in C medium (Roberts *et al.*, 1957) with 0.4% glucose and harvested during exponential growth. To prepare cell-free extracts cells were harvested by centrifugation, washed three times with TKMM buffer, and finally resuspended in TKMM and passed through a French pressure cell at 8000 psi.

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† Preliminary note: Nepokroeff and Aronson (1967).

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[1] Abbreviations used are: Fd, ferredoxin; TKMM buffer, 0.01 M Tris–0.01 M KCl–0.01 M MgOAc–0.006 M 2-mercaptoethanol (pH 7.8).