Isolation of Two Lipolytic Pituitary Peptides*

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ABSTRACT: Pig pituitary glands were extracted with 70% acetone at pH 1.1, acetone was added to a concentration of 92%, and the resulting 70–92% acetone precipitate was dissolved in 0.1 N acetic acid and adsorbed with oxycellulose to remove adrenocorticotropin and melanocyte-stimulating hormones. To the unadsorbed supernatant solution, trichloroacetic acid was then added to a concentration of 5%. The resulting 5% trichloroacetic acid precipitate (labeled fraction 7) possesses lipolytic activity *in vitro* on rabbit, guinea pig, chicken, and pigeon adipose tissue, and raises plasma free fatty acid concentration in the anesthetized monkey. By gel filtration of fraction 7 on Sephadex G-75, followed by ion-exchange chromatography on carboxymethylcellulose, two lipolytic peptides (labeled peptides 7D6 and 7D7) were isolated in homogeneous form. Peptide 7D6 in the amount recovered accounted for about 60% of the lipolytic activity of fraction 7, and peptide 7D7 for about 20%. The minimal effective dose for the lipolytic activity of 7D6 on rabbit adipose tissue *in vitro* was 0.1 μg/ml, and of 7D7 0.01 μg/ml. Both peptides were active as well on the adipose tissue of guinea pig and chicken, and inactive on that of rat, hamster, cat, and opossum. As little as 0.01 mg of 7D6 increased the plasma free fatty acid concentration in the intact rabbit, and as little as 2.5 mg had this effect in the monkey. The rise in plasma free fatty acid level was associated with an increase in blood glucose concentration and a fall in plasma total amino acid level. Molecular weight of 7D6 was estimated by sedimentation equilibrium as 8900, and that of 7D7 as 5500. The differing amino acid compositions of 7D6 and 7D7 are reported.

Peptide 7D6, porcine fraction L, and Astwood's porcine peptide II are closely similar or identical in amino acid composition, electrophoretic mobility, molecular weight, and lipolytic potency and appear to be the same peptide. Peptides 7D6 and 7D7 resemble the "neurophysin" group of peptides in amino acid composition.

During the past 10 years, three laboratories have reported on novel pituitary peptides which are highly active lipolytic agents on rabbit adipose tissue, but virtually inactive on the rat tissue. (1) This laboratory described a fraction of pig pituitary, labeled fraction H (Rudman *et al.*, 1960), and, in more purified form, fraction L (Rudman *et al.*, 1961), which was lipolytic *in vitro* and *in vivo* in the rabbit but not in the rat. These preparations were weakly active on guinea pig adipose tissue, but inactive on the tissues of mouse, hamster, dog, and pig (Rudman *et al.*, 1962). (2) Astwood and collaborators (Astwood *et al.*, 1961) isolated from pig pituitaries two peptides labeled I and II, both of which were active on the rabbit but not on the rat tissue. Peptide II was found by electrophoretic and immunologic tests to be identical with the major component in fraction H (Friesen *et al.*, 1962). (3) Li and colleagues have isolated three novel lipolytic peptides from sheep, pig, and human pituitaries, labeled fraction L′ (Birk and Li, 1964), β-lipotropin (Li *et al.*, 1965; Graf and Cseh, 1968; Cseh *et al.*, 1968), and γ-lipotropin (Chretien and Li, 1967), all highly active on rabbit adipose tissue but only weakly so on the rat tissue.

Recently Dr. J. D. Fisher of Armour Pharmaceutical Co. called our attention to the fact that a side product in isolation of ACTH[1] and the MSH's from pig pituitaries at Armour possessed considerable lipolytic activity in the rabbit. This side product was labeled "fraction 7"; Dr. Fisher kindly made available to us 30 g of this material. In this report, we describe: (1) isolation from fraction 7 of two peptides with lipolytic activity in the rabbit; (2) characterization of the two peptides by disc electrophoresis, amino acid composition, molecular weight, and assay for lipolytic activity in seven other species besides the rabbit. On the basis of these characteristics, we then discuss the probable relationship of these two peptides to the previously identified lipolytic pituitary peptides.

Materials and Methods

"Fraction 7" was generously donated by Dr. Fisher. This material was prepared from pig pituitary glands as follows. The frozen glands were extracted in 70% acetone (pH 1.1) according to Lyons (1937). Acetone was added to the extract up to a concentration of 92%, and the precipitate (fraction 2) and supernatant solution (fraction 3) were collected (Lyons, 1937). Fraction 2 was dissolved in 0.1 N acetic acid and treated with oxycellulose according to Astwood *et al.* (1951). The 0.1 N HCl eluate, containing ACTH and α- and β MSH, is fraction 3. The unadsorbed supernatant of the first oxycellulose step (fraction 4) was treated again with oxycellulose; the 0.1 N HCl eluate is fraction 5, and the 0.1 N acetic acid supernatant of the second adsorption is fraction 6. To this solution at 0° was

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[1] Abbreviations used are: ACTH, adrenocorticotropin; MSH, melanocyte-stimulating hormone; TSH, thyroid-stimulating hormone; FFA, free fatty acid.

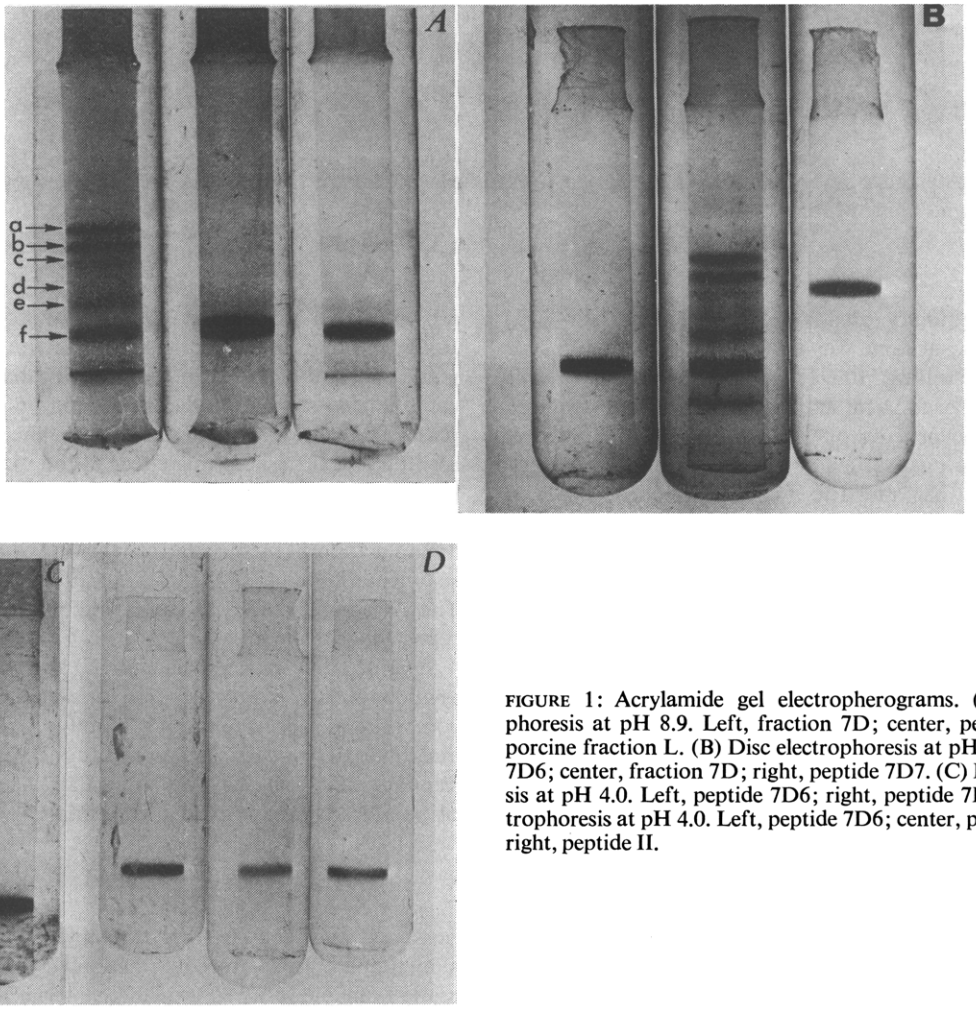

FIGURE 1: Acrylamide gel electropherograms. (A) Disc electrophoresis at pH 8.9. Left, fraction 7D; center, peptide 7D6; right, porcine fraction L. (B) Disc electrophoresis at pH 8.9. Left, peptide 7D6; center, fraction 7D; right, peptide 7D7. (C) Disc electrophoresis at pH 4.0. Left, peptide 7D6; right, peptide 7D7. (D) Disc electrophoresis at pH 4.0. Left, peptide 7D6; center, porcine fraction L; right, peptide II.

added trichloroacetic acid to a concentration of 5 g/100 ml. The precipitate, fraction 7, was dialyzed and lyophilized.

Oxycellulose-purified porcine ACTH (100–120 units/mg) was purchased from Wilson Laboratories, and porcine βMSH was a gift from Dr. S. Lande of Yale University. Bovine TSH was an Armour product. L-Epinephrine bitartrate and crystalline bovine glucagon were purchased from Mann Research Labs. A few milligrams of porcine fraction L prepared 7 years earlier (Rudman *et al.*, 1961), and of porcine peptide II prepared as described by Friesen and Astwood (1967) and generously furnished 3 years ago by Dr. E. B. Astwood, were still available for a limited number of experiments in the present study.

Male animals of these species were used: chinchilla rabbits (3–4.5 kg), Wistar rats (100–150 g), Syrian hamsters (150–175 g), albino guinea pigs (500–750 g), carnot pigeons (500–800 g), domestic chickens (500–1000 g), cats (3–4 kg), and Virginia opossums (3–6 kg). Five female *Macaca mulatta* monkeys (4.0–6.6 kg) were also employed. Rats and hamsters were fed Purina Laboratory checkers; rabbits and guinea pigs, Purina rabbit chow; pigeons and chickens, cracked corn; opossums, moistened Laddy Boy dog food; cats, commercial cat food; monkeys, fruits, vegetables, and Purina Old World Monkey Chow. Food and water were available to the animals at all times. Except for the monkeys, animals were anesthetized with pentobarbital and sacrificed by cervical dislocation for *in vitro* assays with adipose tissue slices or free fat cells. Lipolytic activity was studied *in vivo* in monkeys by injecting the test material intravenously into animals anesthetized with phencyclidine-HCl (1 mg/kg intramuscularly). The monkeys were under anesthesia for 30 min before the first blood sample was taken and the experimental sample, dissolved in 1 ml of 0.9% NaCl, was injected. The animals remained anesthetized during the following 90 min of the experiment.

Assays for lipolytic activity were done on slices of epididymal (rat, hamster, and guinea pig) or perirenal (rabbit, cat, pigeon, chicken, and opossum) adipose tissue, and in some cases on the free fat cells prepared from epididymal, perirenal, or omental adipose tissue. The techniques have been described previously (Rudman and Del Rio, 1969). Plasma glucose concentration was measured according to Shannon *et al.* (1941), and plasma amino nitrogen by the method of Frame and coworkers (1943).

Gel filtration on Sephadex G-75 was performed at 25° on 4 × 200 cm columns in 1.0 N acetic acid, at a flow rate of 50–70 ml/hr. Effluent was collected in 10-ml fractions; 0.2-ml aliquots were dried at 80° *in vacuo*, and after alkaline hydrolysis analyzed by the colorimetric ninhydrin procedure (Hirs *et al.*, 1956). Peaks of ninhydrin-reactive material were recovered by lyophilization. Ion-exchange chromatography

TABLE I: Summary of *in Vitro* Assays for Lipolytic Activity.[a]

	Yield (mg)[b]	Rat	Hamster	Guinea Pig	Rabbit	Cat	Opossum	Chicken	Pigeon
Porcine fraction 7	1000	0[d]	0	10	1	0	0	10	10
Porcine fraction 7A	70				10				
Porcine fraction 7B	150				10				
Porcine fraction 7C	200				1				
Porcine fraction 7D	300				1				
Purified porcine fraction 7D	177				1				
Porcine 7D1	6.0				0				
Porcine 7D2	2.0				0				
Porcine 7D3	4.5				0				
Porcine 7D4	5.0				0				
Porcine 7D5	25.4				0				
Porcine 7D6	56.5	0	0	10	0.1	0	0	1	
Porcine 7D7	2.0	0	0	1	0.01	0	0	0.1	
Porcine ACTH		0.1	0.1	1	0.1	0	1	0	
Porcine βMSH		0	0	0.1	0.01	0	01	0	
Bovine TSH		0.1	0	1	0	0	0		
Bovine glucagon		1	0	0	0	0	0	0.1	0.01
Epinephrine		0.01	0.01			0	+		+
Porcine fraction L		0	0	+	+[c]	0	0	+	

[a] When activity was found at 10 μg/ml and minimum effective dose subsequently determined, the minimum effective dose is given in the table. [b] Per gram of fraction 7. [c] Indicates activity present at 10 μg/ml, but minimum effective dose not measured. [d] Signifies no activity at 10 μg/ml. Each preparation was tested on at least two different animals from the given species.

was carried out at 25° on a 2 × 30 cm column of carboxymethyl cellulose (CM-32, Reeve-Angel) washed successively with 1.0 N HCl, 1.0 N NaOH, and H_2O, during which fines were removed, and finally equilibrated with 0.02 M ammonium acetate solution (pH 4.0). Elution was performed with a nine-chamber varigrad containing in each chamber 400 ml of one of the following ammonium acetate solutions: chamber 1, 0.02 M, pH 4.0; chamber 2, 0.04 M, pH 4.3; chamber 3, 0.06 M, pH 4.5; chamber 4, 0.08 M, pH 4.8; chamber 5, 0.10 M, pH 5.0; chamber 6, 0.12 M, pH 5.3; chamber 7, 0.15 M, pH 5.5; chamber 8, 0.18 M, pH 6.0; and chamber 9, 0.20 M, pH 7.0. Effluent (3200 ml) was collected in 10-ml fractions with flow rate maintained at 80 ml/hr by means of a peristalsis pump. Each fraction (1.0 ml) was analyzed for protein by the method of Lowry *et al.* (1951). Fractions constituting a peak of Lowry-reactive material were pooled and lyophilized, and then freed of residual ammonium acetate by gel filtration in 1.0 N acetic acid on a 1 × 100 cm column of Sephadex G-10; the peptide was located in the effluent by drying 0.2-ml aliquots of each 3-ml fraction at 80° *in vacuo* and analyzing the residue by the colorimetric ninhydrin procedure after alkaline hydrolysis. The ninhydrin-reactive material was then recovered in salt-free form by lyophilization.

Electrophoresis in polyacrylamide gel was done at pH 8.9, 4.0, and 2.9 in a Büchler "polyanalyst" apparatus. Compositions of the buffers and gels were those of Ornstein and Davis (1962). The sample consisted of 0.5 mg of peptide dissolved in 0.5 ml of upper buffer containing 50 mg of sucrose. Electrophoresis was done at 15° at 2.5 mA/tube for 60 min. Gels were stained in 0.25% Amido Schwarz in 7% acetic acid for 1 hr and destained at 5 mA/tube in 7% acetic acid.

For measurement of amino acid composition, duplicate samples of 1–2 mg of peptide were hydrolyzed in 6 N HCl at 110° for 24 hr. The hydrolysates were taken to dryness on a rotary evaporator at 40° and analyzed in a Beckman-Spinco 120C instrument with the "4-hr hydrolysate buffer system" (Beckman 120C Procedures Manual). Half-cystine and methionine were determined as cysteic acid and methionine sulfone on duplicate 24-hr hydrolysates of performic acid oxidized peptide; a 94% recovery of cysteic acid was assumed (Moore, 1963). Calculation of the content of half-cystine and methionine was based on the ratio of cysteic acid or of methionine sulfone to leucine recovered in the same analysis of oxidized peptide and on the known content of leucine in the unoxidized peptide. Tryptophan, destroyed by acid hydrolysis, was determined by method B of Spies and Chambers (1949). The composition was expressed as g of each amino acid per 100 g of peptide, calculated as described by Margolis and Langdon (1966).

Molecular weight of purified peptides was determined by sedimentation equilibrium (Yphantis, 1964). Molecular weight of certain purified peptides was also estimated from gel filtration in 1 N acetic acid through a 1 × 100 cm column of Sephadex G-75 calibrated (Andrews, 1964) with purified peptides or proteins of known molecular weight.

Results

Fraction 7 was active on rabbit adipose tissue slices with a minimal effective dose of 1.0 μg/ml. It was also active as a lipolytic agent on the free fat cells or adipose tissue slices of guinea pig, chicken, and pigeon, with minimal effective dose

TABLE II: Results of Lipolytic Assays with Adipose Tissue Slices (in Microequivalents of Free Fatty Acids per Gram of Tissue).[a]

Test Substance (Dose, μg/ml)	Rat	Hamster	Guinea Pig	Rabbit	Cat	Opossum	Chicken
None	2.0 ± 0.3	1.2 ± 0.1	1.3 ± 0.1	0.4 ± 0.1	1.4 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
Fraction 7 (10)	1.9 ± 1.1	1.2 ± 0.1	3.7 ± 0.2	11.6 ± 1.0			2.6 ± 0.3
Fraction 7 (1)	1.8 ± 0.2	1.3 ± 0.1	1.2 ± 0.2	3.2 ± 0.2			1.0 ± 0.2
Fraction 7 (0.1)				0.4 ± 0.1			1.0 ± 0.1
Fraction 7 (0.01)							
Peptide 7D6 (10)			6.5 ± 0.3	12.6 ± 1.4	1.3 ± 0.2	1.2 ± 0.1	2.5 ± 0.1
Peptide 7D6 (1)			1.3 ± 0.1	10.5 ± 1.0		1.1 ± 0.2	2.0 ± 0.0
Peptide 7D6 (0.1)			1.7 ± 0.1	5.3 ± 0.4			1.3 ± 0.1
Peptide 7D6 (0.01)				0.8 ± 0.1			1.1 ± 0.2
Peptide 7D7 (10)			9.9 ± 1.0	14.5 ± 1.1	1.1 ± 0.1	1.0 ± 0.1	2.5 ± 0.2
Peptide 7D7 (1)			5.1 ± 0.6	12.7 ± 1.3		1.1 ± 0.1	2.4 ± 0.2
Peptide 7D7 (0.1)			1.2 ± 0.1	11.3 ± 1.0			2.1 ± 0.1
Peptide 7D7 (0.01)			1.3 ± 0.1	6.5 ± 0.4			1.5 ± 0.3
Peptide 7D7 (0.001)				0.3 ± 0.0			1.0 ± 0.1
ACTH (10)	11.9 ± 1.5	15.2 ± 1.4	9.2 ± 0.5	10.7 ± 1.0	1.5 ± 0.3	3.7 ± 0.3	1.2 ± 0.2
ACTH (1)	8.6 ± 1.1	7.2 ± 0.6	2.5 ± 0.4	7.5 ± 1.0		2.5 ± 0.2	1.2 ± 0.2
ACTH (0.1)	5.9 ± 0.8	3.5 ± 0.3	1.1 ± 0.2	3.9 ± 0.3		1.2 ± 0.2	1.1 ± 0.1
ACTH (0.01)	1.9 ± 0.2	1.1 ± 0.1		0.6 ± 0.1		1.1 ± 0.1	1.3 ± 0.3
βMSH (10)	1.8 ± 0.1	1.3 ± 0.2	11.0 ± 0.8	14.0 ± 0.1	1.2 ± 0.2	3.4 ± 0.2	0.9 ± 0.1
βMSH (1)	2.1 ± 0.2	1.1 ± 0.4	9.0 ± 0.9	13.6 ± 1.2		3.3 ± 0.3	1.4 ± 0.3
βMSH (0.1)			4.3 ± 0.4	11.9 ± 0.8		2.7 ± 0.2	1.0 ± 0.2
βMSH (0.01)			1.7 ± 0.2	5 ± 0.3		1.5 ± 0.2	
βMSH (0.001)			1.1 ± 0.1	0.8 ± 0.1			
Glucagon (10)	9.3 ± 1.0	1.4 ± 0.4	1.2 ± 0.2	0.3 ± 0.1		1.0 ± 0.1	12.3 ± 0.9
Glucagon (1)	7.5 ± 0.5	1.2 ± 0.3	1.1 ± 0.1	0.4 ± 0.1			6.7 ± 0.7
Glucagon (0.1)	3.1 ± 0.3						4.0 ± 0.3
Glucagon (0.01)	2.5 ± 0.3						1.4 ± 0.3
Epinephrine (10)			1.5 ± 0.3	0.5 ± 0.2	1.0 ± 0.1	3.3 ± 0.2	1.6 ± 0.1
Epinephrine (1)	12.4 ± 1.0	17 ± 2.3	1.4 ± 0.2	0.4 ± 0.1		2.7 ± 0.2	
Epinephrine (0.1)	11.0 ± 0.7	15 ± 1.1				1.1 ± 0.1	
Epinephrine (0.01)	5.5 ± 0.3	6 ± 0.5					
Epinephrine (0.001)	1.2 ± 0.1	1.0 ± 0.1					
Porcine L (10)	2.7 ± 0.1	1.4 ± 0.1	6.3 ± 0.4	11.6 ± 1.1	1.3 ± 0.1	1.5 ± 0.1	2.1 ± 0.3

[a] In the course of this study, each agent was tested on the adipose tissue of two to six animals of each species. Because of the limitations of space, this table gives the results of a single assay for each lipolytic substance in each species. Most values in the table represent average plus and minus standard error of four observations, since each dose was tested in quadruplicate in a single assay. Values when test substance was not added (control flasks) are average plus and minus standard error for 12–16 observations, since these values are averages of all control data for those two or three assays summarized in each vertical column of the table.

of 10 μg/ml in each case, but inactive at the 10-μg/ml concentration on the tissue slices of rat, hamster, cat, and opossum. The results with adipose tissue slices are summarized in Tables I and II; parallel findings were obtained with free fat cells. Disc electrophoresis (Figure 1) of fraction 7D at pH 8.9 showed the presence of three major bands (labeled a, b, and f) and three minor bands (labeled c, d, and e).

Fractionation of fraction 7 began with gel filtration of 200 mg dissolved in 10 ml of 1.0 N acetic acid on a 4 × 200 cm column of Sephadex G-75. Four fractions (A–D) of ninhydrin-reactive material were recovered by lyophilization (Figure 2A). All stimulated lipolysis in rabbit adipose tissue. Fraction 7C,D was the most potent, with a minimal effective dose (1 μg/ml) of ten times less than that of 7A,B. Fraction 7D was selected for further investigation in the present study. By gel filtration of a total of 1 g of fraction 7 (in batches of 200 mg, as shown in Figure 2A), a total of 300 mg of fraction 7D was prepared. This material was passed a second time through the Sephadex G-75 column, with recovery of 177 mg of "purified" 7D (Figure 2B); 100 mg of purified 7D was dissolved in 10 ml of 0.02 M ammonium acetate buffer (pH 4.0) and fractionated by cation-exchange chromatography on CM-32. Seven peaks of Lowry-reactive material were obtained, labeled 7D1 → 7D7 (Figure 3). The sum of the yields of these seven peaks amounted to 58% of the quantity of purified

TABLE III: Effect of Intravenous Injection of Fraction 7, Peptide 7D6, ACTH, and βMSH on the Plasma FFA (μequiv/l.), Plasma Glucose (GL) (mg/100 ml), and Plasma α-Amino Nitrogen (AAN) (mg/100 ml) Concentrations of the Rabbit and Monkey.

Animal (No. Tested)	Material Injected (mg)	Time after Injection											
		0 min			30 min			60 min			90 min		
		FFA	GL	AAN	FFA	GL	AAN	FFA	GL	AAN	FFA	GL	AAN
Rabbit (8)	None	410 ± 30	75 ± 6	8.7 ± 0.3	430 ± 28	80 ± 6	8.9 ± 0.9	420 ± 55	82 ± 4	8.5 ± 0.3	450 ± 56	85 ± 7	
Rabbit (3)	Fraction 7 (2.5)	340 ± 58	81 ± 6	9.7 ± 0.5	2940 ± 540	162 ± 10	7.0 ± 0.5	3110 ± 330	135 ± 10	7.8 ± 0.6	2930 ± 300	110 ± 11	
Rabbit (3)	Fraction 7 (1.0)	370 ± 45	85 ± 5		3110 ± 580	148 ± 15		2750 ± 350	129 ± 11		3040 ± 270	90 ± 6	
Rabbit (3)	Fraction 7 (0.1)	310 ± 23	78 ± 10		640 ± 90	167 ± 18		620 ± 70	115 ± 8				
Rabbit (3)	Fraction 7 (0.05)	440 ± 31	80 ± 7		340 ± 38	85 ± 7		310 ± 35	82 ± 7				
Rabbit (3)	Peptide 7D6 (1.0)	480 ± 25	75 ± 6	9.6 ± 0.5	2980 ± 115	148 ± 25	7.7 ± 0.8	2430 ± 450	136 ± 11	6.9 ± 0.8			
Rabbit (3)	Peptide 7D6 (0.5)	450 ± 43	81 ± 7	8.7 ± 0.4	3310 ± 320	152 ± 30	7.1 ± 0.5	2870 ± 210	129 ± 8	6.5 ± 0.6			
Rabbit (3)	Peptide 7D6 (0.10)	410 ± 55	82 ± 4		2840 ± 250	134 ± 16							
Rabbit (3)	Peptide 7D6 (0.05)	520 ± 39	78 ± 8		2010 ± 280	130 ± 16							
Rabbit (3)	Peptide 7D6 (0.01)	390 ± 48	75 ± 9		1840 ± 200	150 ± 12							
Rabbit (3)	Peptide 7D6 (0.001)	280 ± 26	90 ± 5		450 ± 60	115 ± 8							
Rabbit (3)	ACTH (1.0)	277 ± 35	101 ± 5	9.0 ± 1.0	2847 ± 310	212 ± 24	7.8 ± 0.9	2671 ± 290	237 ± 26	8.1 ± 0.7			
Rabbit (3)	ACTH (0.5)	302 ± 27	94 ± 11		2444 ± 330	144 ± 10		2142 ± 260	179 ± 16				
Rabbit (3)	ACTH (0.10)	252 ± 35	74 ± 6		3049 ± 250	179 ± 18		2998 ± 310	169 ± 12				
Rabbit (3)	ACTH (0.05)	560 ± 50	85 ± 7		2381 ± 210	122 ± 14							
Rabbit (3)	ACTH (0.01)	410 ± 37	87 ± 8		1304 ± 90	100 ± 6							
Rabbit (3)	ACTH (0.001)	380 ± 38	76 ± 5		350 ± 40	81 ± 7							
Rabbit (3)	βMSH (1.0)	290 ± 25	85 ± 7	8.8 ± 0.3	3750 ± 470	165 ± 20	6.7 ± 0.7						
Rabbit (3)	βMSH (0.5)	460 ± 40	90 ± 8		2250 ± 180	171 ± 21							
Rabbit (3)	βMSH (0.10)	310 ± 39	76 ± 7		2900 ± 290	153 ± 19							
Rabbit (3)	βMSH (0.05)	390 ± 18	84 ± 8		2381 ± 200	147 ± 20							
Rabbit (3)	βMSH (0.01)	350 ± 15	75 ± 9		2650 ± 210	155 ± 11							
Rabbit (3)	βMSH (0.001)	290 ± 20	90 ± 10		222 ± 20	97 ± 7							
Monkey (4)	None	295 ± 40	82 ± 10	8.9 ± 1.0	310 ± 35	85 ± 9	8.7 ± 0.7	330 ± 45	81 ± 11	8.5 ± 0.6	315 ± 38	87 ± 10	
Monkey (1)	Peptide 7D6 (5)	370	90	8.6	866	174	6.2	601	170	6.1			
Monkey (1)	Peptide 7D6 (5)	448	95	8.8	892	114	7.4	709	195	6.3			
Monkey (1)	Peptide 7D6 (5)	318	85	8.9	996	245	7.2	683	123	6.0			
Monkey (1)	Peptide 7D6 (2.5)	418	89	9.7	992	121	6.5	809	129	6.3			
Monkey (1)	Peptide 7D6 (2.5)	526	103	9.2	1205	185	7.9	1153	124	7.2			
Monkey (1)	Peptide 7D6 (2.5)	580	69	8.9	1210	79	6.6	1270	112	5.5			
Monkey (1)	Peptide 7D6 (2.5)	605	75	9.6	1550	95	6.7	1530	114	6.0			

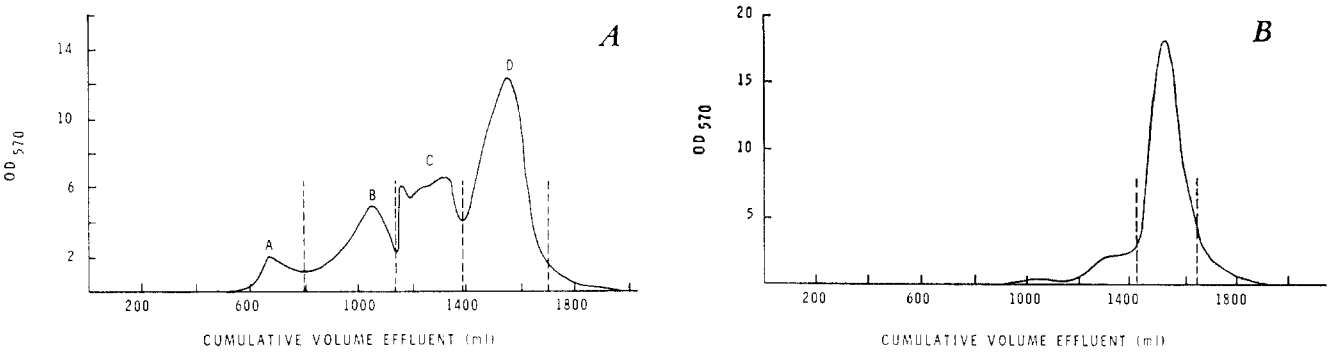

FIGURE 2: Gel filtration. (A) Of 500 mg of fraction 7 on 4 × 200 cm column of G-75 in 1.0 N acetic acid. Tubes labeled A, B, C, and D were pooled and lyophilized to give fractions 7A–7D, respectively. (B) Of 200 mg of fraction 7D by gel filtration on a 4 × 200 cm column of G-75 in 1.0 N acetic acid. Ordinate shows optical density measured at 570 mμ following reaction of alkaline hydrolysate of 1 ml of effluent with ninhydrin.

fraction 7D applied to the column (Table I). Only 7D6 and 7D7 stimulated lipolysis in the rabbit's adipose tissue, the minimal effective doses for this action being 0.1 and 0.01 μg per ml, respectively. Both 7D6 and 7D7 moved as a single band on disc electrophoresis at 8.9, 4.0, and 2.9 (Figure 1). Fraction 7D6 corresponded to band f in fraction 7D, and 7D7 to band b. On disc electrophoresis at pH 8.9, 4.0, and 2.9, porcine fraction L and porcine peptide II both exhibited one major band which had the same mobility as 7D6; at pH 8.9, two to three additional faint bands could be discerned in porcine L, and in peptide II.

Peptides 7D6 and 7D7, porcine fraction L, and fraction 7 showed the same pattern of species variation in their lipolytic action: activity on the tissue or free fat cells of rabbit, guinea pig, and chicken; inactivity on the tissue or free cells of rat, hamster, cat, and opossum. Peptide 7D7 was ten times more potent than 7D6 in all three species responsive to these peptides (rabbit, guinea pig, and chicken). Each of the other lipolytic agents tested was likewise active in some species and inactive in others. The patterns of species variability for ACTH, βMSH, TSH, glucagon, and epinephrine were different from each other, and different from the common pattern exhibited by 7D6, 7D7, and porcine L.

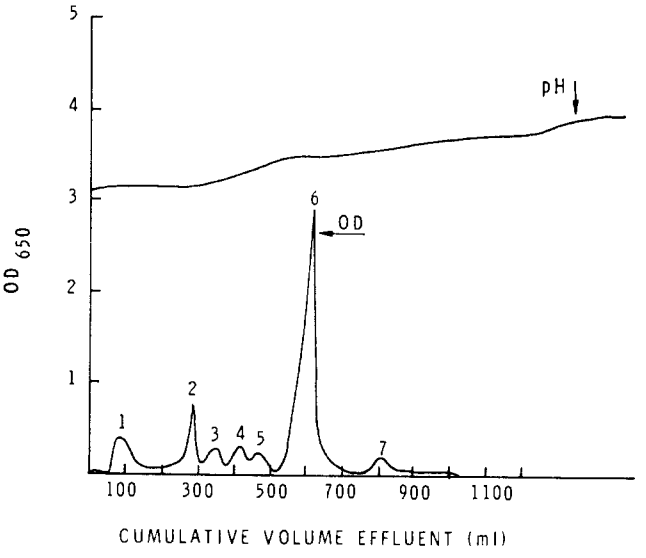

FIGURE 3: Chromatography of 100 mg of purified fraction 7D on 2 × 30 cm column of CM-32. Ordinate shows optical density of 1 ml of effluent in the Lowry analysis.

Intravenous injection of fraction 7 or peptide 7D6 into rabbits at doses ranging between 0.01 and 2.5 mg (dissolved in 1 ml of 0.9% NaCl) caused a five times or greater increase in plasma free fatty acid concentration within 30 min; this increase was still present at 60 min (Table III). A similar effect was produced by ACTH and βMSH. The minimal effective doses for this *in vivo* lipolytic effect were: fraction 7, 100 μg; 7D6, ACTH and βMSH, 10 μg. Fortuitously, concentrations of plasma glucose and plasma α-amino nitrogen were measured in one rabbit injected with 2.5 mg of fraction 7; glucose was increased by 80% at 60 min and the total of amino acids was reduced by 30%. Further studies showed that fraction 7, peptide 7D6, ACTH, and βMSH regularly produced hyperglycemia and hypoaminoacidemia, as well as increase in plasma free fatty acid, in the intact rabbit. These changes were evident at 30 min and still present at 90 min. The minimal effective doses for the hyperglycemic effect of these preparations (10–100 μg) were similar or identical with those for the *in vivo* lipolytic effect (Table III); the data on the reduction of plasma amino acids were not sufficiently detailed to define the minimal effective dose for this effect. In the anesthetized monkey, 5 or 2.5 mg of 7D6 caused an increase in plasma concentrations of free fatty acid and glucose, and a fall in that of amino acid nitrogen, in the majority of animals tested (Table III).

Amino acid compositions of 7D6 and 7D7 are given in Table IV, where those of other pituitary lipolytic peptides and of certain relevant posterior lobe peptides of known composition are also shown for comparison. Notable is the close resemblance in amino acid composition of 7D6 and the porcine posterior lobe peptide of Wuu and Saffran (1969); 7D6, 7D7, and the Wuu–Saffran peptide have in common a substantial content of half-cystine (absent in βMSH, ACTH, β-lipotropin, γ-lipotropin, and ovine L′) and absence of tryptophan (present in the latter five lipolytic peptides). The amino acid composition of porcine L and peptide II, although faint minor components were visualized in these two preparations by disc electrophoresis, was also measured and found closely similar to that of 7D6.

Partial specific volumes of 7D6 and 7D7 were calculated

TABLE IV: Amino Acid Compositions.[a]

	Porcine 7D6	Porcine Fraction L	Porcine Peptide II	Porcine 7D7	Porcine ACTH	Porcine βMSH	Ovine L′	Ovine β-Lipotropin	Ovine γ-Lipotropin	Bovine Preddie Peptide	Porcine Wuu–Saffran Peptide	Bovine Neurophysin I	Bovine Neurophysin II
Aspartic acid	6.5	6.6	6.7	7.7	5.1	10.7	5.3	4.6	3.6	6.5	6.4	8.1	5.9
Threonine	2.2	2.3	2.2	2.3	0	0	4.7	4.1	1.6	2.0	2.2	2.4	2.1
Serine	5.8	5.9	5.6	5.3	3.8	4.0	4.0	4.4	4.1	6.7	7.0	5.6	5.3
Glutamic acid	20.0	20.5	19.9	20.0	14.2	12.0	19.8	20.8	26.5	14.7	18.6	14.6	17.1
Proline	7.7	7.5	7.7	7.5	8.5	13.5	6.0	4.9	6.1	7.8	6.7	10.0	8.5
Glycine	8.1	8.0	8.4	6.6	3.8	5.3	4.4	4.6	4.5	10.1	8.6	8.5	8.6
Alanine	6.4	6.6	6.4	7.1	4.7	0	8.7	9.3	12.3	4.7	5.4	5.8	4.1
Valine	2.2	2.2	2.3	2.5	6.5	0	3.0	2.1	1.6	4.1	2.2	2.9	3.9
Half-cystine	12.6	12.4	12.5	7.5	0	0	0	0	0	10.1	12.9	13.1	14.3
Methionine	1.5	1.4	1.4	1.9	2.9	6.1	2.0	2.6	2.1	0	1.4	0.5	1.3
Isoleucine	2.3	2.0	2.2	1.8	0	0	1.7	1.1	0	2.3	2.5	2.3	2.3
Leucine	7.6	7.7	7.7	7.7	5.0	0	6.9	6.8	7.1	6.6	8.9	7.0	6.6
Tyrosine	1.8	1.6	1.7	2.3	7.2	7.6	5.0	4.9	5.1	0	1.7	2.7	1.9
Phenylalanine	4.4	4.4	4.3	4.3	9.7	6.8	4.5	4.4	2.3	3.3	4.6	4.3	4.5
Lysine	3.6	3.3	3.4	5.8	11.3	11.9	11.8	12.9	8.1	2.4	2.7	3.1	2.7
Histidine	0	0	0	1.4	3.0	6.4	2.1	2.8	2.2	0	0	1.4	0
Arginine	7.4	7.5	7.6	8.6	10.3	7.2	7.2	7.9	9.9	10.5	8.2	7.6	10.5
Tryptophan	0	0	0	0	4.1	8.6	2.9	1.9	2.9	8.2	0	0	0
Molecular weight	8,900			5,500	4,567	1,382	5,400	9,900	6,000	5,500	9,900	19,000	21,000

[a] Expressed as g of each residue/100 g of peptide, of porcine peptide 7D6, porcine 7D7, porcine fraction L (Rudman *et al.*, 1961), porcine peptide II (Astwood *et al.*, 1961), porcine ACTH, porcine βMSH, ovine fraction L′ (Birk and Li, 1964), ovine β-lipotropin (Li *et al.*, 1965), ovine γ-lipotropin (Chretien and Li, 1967), the bovine neurohypophyseal peptide of Preddie (1965), the porcine neurohypophyseal peptide of Wuu and Saffran (1969), and the bovine neurophysins of Hollenberg and Hope (1968).

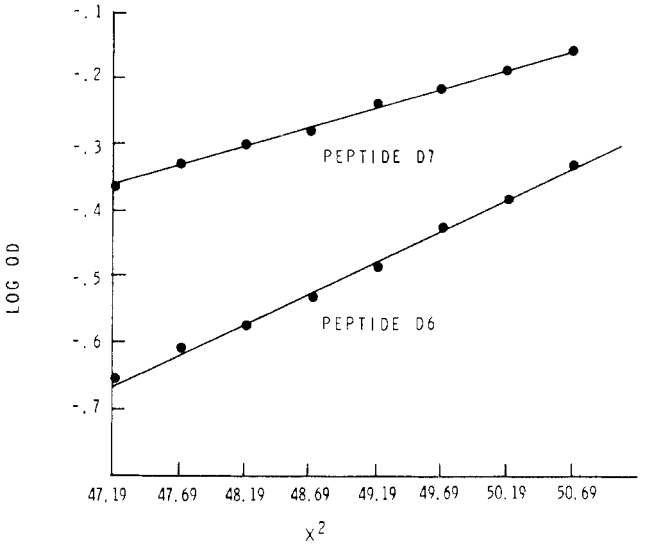

FIGURE 4: Plot of log of concentration (optical density at 280 mμ) against the square of the distance from the center of rotation derived from sedimentation equilibrium at 20,000 rpm of peptides 7D6 and 7D7.

from the amino compositions to be 0.717 and 0.733, respectively (Cohn and Edsall, 1943). Molecular weights of 7D6 and 7D7 were determined by sedimentation equilibrium in water to be approximately 8900 and 5500, respectively (Figure 4). Peptide 7D6, porcine fraction L, and peptide II emerged at the same cumulative volume on gel filtration through a 1 × 100 cm column of Sephadex G-75 in 1.0 N acetic acid (peak optical density at 58–62 ml); calculation of molecular weight from this elution volume gave a value of 10,000–12,000 for all three peptide preparations.

Discussion

Fraction 7 contains at least two peptides with lipolytic activity. 7D6 is a major electrophoretic component of fraction 7, constitutes at least 32% of this fraction, has a molecular weight of about 8900, and is equipotent with ACTH and one-tenth as potent as βMSH as a lipolytic agent on rabbit adipose tissue *in vitro*. 7D7 is a minor component of fraction 7, constituting about 1% of the latter, has a molecular weight of approximately 5500, is ten times more potent than ACTH, and is equipotent with βMSH on the rabbit tissue. Among seven lipolytic agents examined, only 7D6 and 7D7 showed an identical pattern of species variation in lipolytic potency. Furthermore 7D6 and 7D7 show a general resemblance in amino acid composition (Table IV). These two peptides therefore may be structurally related. It is likely that other lipolytic peptides of larger molecular weight are also present in fraction 7, since fractions 7A, 7B, and 7C, containing materials of progressively higher molecular weight than 7D, all possess detectable lipolytic activity. If one defines a unit of lipolytic activity as the smallest activity per ml of incubation medium to which the rabbit tissue slice will respond, then 1 g of fraction 7 contains about 1,000,000 units. The quantity of 7D6 (57 mg) isolated from 1 g of fraction 7 accounts for about 570,000 units, and that of 7D7 (2 mg), accounts for about 200,000 units. Although other active components may be present in fraction 7, these calculations suggest that most of the lipolytic activity of this preparation can be accounted for by its content of 7D6 and 7D7.

What is the relation of 7D6 and 7D7 to the numerous previously isolated lipolytic peptides of the pituitary? Porcine 7D6 and porcine L exhibited identical electrophoretic mobilities, patterns of lipolytic species specificity, and elution volumes from Sephadex G-75, and closely similar values for amino acid composition, and therefore appear to represent the same pituitary peptide isolated by two different techniques. Porcine fraction L had already been shown to be identical with peptide II (Friesen *et al.*, 1962); this earlier conclusion is supported by the present finding of identical electrophoretic mobility of these two peptides in acrylamide gel at pH 8.9, 4.0, and 2.9, identical elution volume from Sephadex G-75, and closely similar values for amino acid composition. This reduces the complexity of the situation somewhat to the question, what is the relationship between 7D6 (or fraction L, or peptide II), 7D7, ACTH, α- and βMSH, peptide I, peptide L′, and β- and γ-lipotropins? The available evidence indicates that ACTH, α- and βMSH, peptide I, peptide L′, and β- and γ-lipotropins are structurally related. Thus ACTH, peptide I, and β- and γ-lipotropins are known to contain the sequence ...Met-Glu-His-Phe-Arg-Trp-Gly... which is intimately related to the lipolytic, melanotropic, and corticotropic properties (Hofmann and Katsoyannis, 1963; Li *et al.*, 1965; Lohmar and Li, 1968). Although the sequence of peptide L′ has not yet been reported, this peptide possesses substantial corticotropic and melanotropic activity in addition to the lipolytic activity (Birk and Li, 1964). All five of these peptides are highly active on the rabbit tissue, but only ACTH is strongly active on that of the rat.

Are 7D6 and 7D7 members of this family? Three types of evidence suggest not.

Location. Peptide 7D6 (peptide II) is located in the posterior lobe (derived from infundibular diverticulum of brain) (Friesen and Astwood, 1967), while ACTH, the MSH's, and β-lipotropin are located in the anterior or intermediate lobes (derived from hypophyseal diverticulum of pharynx) (Li *et al.*, 1966).

Pattern of Species Specificity. 7D6 and 7D7 show a common pattern which is different from that of βMSH or ACTH (Table I).

Amino Acid Composition. As noted above, tryptophan occurs in the sequence common to ACTH, α- and βMSH, peptide I, and β- and γ-lipotropins and is also present in the as yet unsequenced L′. All these peptides lack half-cystine. In contrast, 7D6 and 7D7 are rich in half-cystine and contain no tryptophan. These clues suggest that 7D6 and 7D7 may not be structurally related to the ACTH–MSH–I–L′–β- and γ-lipotropin group.

The glycoprotein TSH shows a different pattern of species variation from 7D6, 7D7, and the other lipolytic pituitary peptides mentioned above. Furthermore, porcine fraction H (the major component of which is identical with 7D6) was shown to be inactive in the TSH assay (Rudman *et al.*, 1961). Therefore there is no reason to suspect that 7D6 and 7D7 may be related to TSH.

Some clues, on the other hand, point to a possible relation of 7D6 and 7D7 to the porcine posterior lobe peptide recently isolated by Wuu and Saffran (1969). First of all, Friesen and

Astwood (1967) showed that peptide II (apparently identical with 7D6) was located in the posterior lobe, decreased markedly in concentration in the posterior lobe extract after the animal was dehydrated, and was present in only low concentration in the posterior lobe of rats with hereditary diabetes insipidus. Peptide II formed a dissociable ionic complex with lysine-vasopressin *in vitro*. Friesen and Astwood accordingly concluded that peptide II was involved in the storage or transport of antidiuretic hormone. They stated that peptide II was rich in cystine and possessed a molecular weight of >25,000, but did not present data for these conclusions. (The present study confirms the high cystine content but indicates a molecular weight of about 8900 for 7D6, apparently identical with peptide II.) In 1968, Hollenberg and Hope isolated from bovine posterior lobes two proteins labeled neurophysin I and II, with mol wt 19,000 and 21,000, respectively, which bind vasopressin and oxytocin. In 1969, Wuu and Saffran isolated a peptide of mol wt 9900 from the pig's posterior lobe. This peptide also was highly active in binding vasopressin and oxytocin; its amino acid composition, marked by a high cystine content and absence of tryptophan, was similar to that of bovine "neurophysin II" of mol wt 21,000. Wuu and Saffran discounted a possible close relation between the substance which they had isolated and peptide II of Astwood and coworkers, because of the estimated molecular weight of over 25,000 of the latter substance (Friesen and Astwood, 1967). But the present data show that both the molecular weight and the values for amino acid composition of 7D6 (or peptide II) are in fact closely similar to those of the Wuu–Saffran peptide. Perhaps 7D6, 7D7, the bovine neurohypophyseal peptide of Preddie (Preddie and Saffran, 1965; Preddie, 1965), the Wuu–Saffran peptide, and neurophysins I and II of Hollenberg and Hope, may be related but distinct members of a series of cystine-rich posterior lobe peptides, with molecular weights ranging from 5,500 to 21,000, some of which bind vasopressin or oxytocin. To answer these questions, it will be necessary to learn whether 7D6 and 7D7 bind these hormones, and to compare the electrophoretic and possible lipolytic properties of all the various posterior lobe peptides under consideration.

Finally it should be mentioned that lipolytic activity on one or another species of fat cells (mammalian or avian) is a common characteristic of several hormones believed to operate through the adenyl cylase → 3,5-cyclic AMP mechanism (ACTH, MSH, TSH, glucagon, and catecholamines) (Robinson *et al.*, 1967). Each of these hormones has as its principal physiologic function some cyclic AMP-mediated action on extra-adipose cell types. Therefore the question arises whether 7D6 and 7D7 may also have other biologic properties besides the lipolytic activity in the rabbit, guinea pig, chicken and monkey, and a possible role in the storage or transport of neurohypophyseal peptides (Friesen and Astwood, 1967). The hyperglycemic and hypoaminoacidemic effects of 7D6 in the rabbit and monkey may be evidence in this direction. To explore this possibility further, it will be necessary to learn whether these effects are secondary to the mobilization of FFA, or whether they represent independent actions of the peptide. Thus, for example, the hyperglycemic effect could result from stimulation by 7D6 of hepatic glycogenolysis, independently of the activation of lipolysis in fat cells. Alternatively, the hyperglycemia could reflect inhibition of glucose metabolism within muscle (Randle *et al.*, 1964) and liver (Porter and Long, 1958) by newly mobilized fatty acids or their coenzyme A esters. The appearance of hyperglycemia during mobilization of FFA in the rabbit injected with ACTH or βMSH strengthens the likelihood of the latter mechanism. It may be possible to distinguish between these two possibilities by determining whether the pattern of species variation for the hyperglycemic effect of 7D6 parallels that of the lipolytic effect, or whether (as in the case of glucagon) potent hyperglycemic activity may obtain in some species which are resistant to the peptide's lipolytic effect. Similar considerations apply to the interpretation of the hypoaminoacidemic effect of peptide 7D6.

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The Isolation and Identification of 7-Oxo-8-aminopelargonic Acid, a Biotin Intermediate*

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ABSTRACT: The identity of a biotin vitamer which was previously shown to be an intermediate in biotin biosynthesis was made possible by its isolation in crystalline form. The purification was carried out by large scale column chromatography of 100 gal of culture medium from *Penicillium chrysogenum*. The compound was shown to be identical with 7-oxo-8-aminopelargonic acid by elemental analysis, mixture melting point, infrared spectrophotometry, and mass spectroscopy. Both the natural and synthetic compounds gave identical growth curves with yeast and were 65–95% as active as biotin.

In an early study with *Phycomyces blakesleeanus* an unknown biotin vitamer was shown to accumulate in the growth medium along with biotin and desthiobiotin (Eisenberg, 1963). Although present in very low concentrations, it was possible to show by electrophoretic analysis that the vitamer contained both an amino group and a carboxyl group with the charges widely separated. In addition the vitamer did not bind to the protein, avidin, indicating the absence of a ureido structure. The very low concentration of the compound precluded its isolation and identification at that time.

It was subsequently found that *Penicillium chrysogenum* produced the same biotin vitamer in much higher yields (Eisenberg, 1966). Additional information about its structure was obtained when it was found that $^{35}SO_4^{2-}$ was not incorporated into the vitamer (Eisenberg, 1965). This observation indicated the absence of the tetrahydrothiophene ring and therefore suggested an open-chain structure for the vitamer. Since an intermediary role in the biosynthesis of biotin was proposed for this vitamer, based on incorporation studies with 1,7-[^{14}C]pimelic acid, the open-chain structure indicated the vitamer was an early intermediate (Eisenberg and Maseda, 1966; Eisenberg, 1966). It was postulated that the condensation of either serine or alanine with pimelic coenzyme A would yield an open-chain compound with the observed properties. Attempts to determine which of the two amino acids was a precursor of the vitamer did not give definite results because incorporation of the labeled amino acids was extremely low. Since the vitamer comprised 60–80% of the total biotin activity of the medium, it was considered feasible that a large scale purification procedure could yield sufficient crystalline material for characterization. The present study describes the isolation of the vitamer in crystalline form by column chromatographic procedures and its identification as 7-oxo-8-aminopelargonic acid.

While this work was in progress, it was shown by Iwahara *et al.* (1965) that a compound with properties similar to those of the unknown vitamer was formed in the medium of *Bacillus sphaericus*. They were able to identify the compound as 7KAP[1] by chromatography in three solvent systems (Iwahara *et al.*, 1966). Soon after, the condensation of pimelic coenzyme A and L-alanine to form 7KAP was demonstrated in cell-free extracts of *Escherichia coli* by Eisenberg and Star (1968).

Materials and Methods

P. chrysogenum was grown in 100 gal of Czapek-Dox medium for 7 days with continuous aeration. The filtrate was pan-dried, yielding about 4 kg of hygroscopic material.

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[1] Abbreviations used are 7KAP for 7-oxo-8-aminopelargonic acid and mmu for millimass unit.