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*TWO METABOLICALLY ACTIVE PEPTIDES FROM PORCINE PITUITARY GLANDS**

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The injection of crude pituitary extract into animals was shown in the 1930's to cause ketosis, increased fat in the liver, and depletion of fat depots leading to the concept of a fat-mobilizing hormone of the pituitary. These and related effects were later ascribed variously to thyrotropin, prolactin, and growth hormone, and more recent tests in mice and rats showed corticotropin to be the most potent agent yet tested.[1] Experiments using the rabbit, however, have revealed both α- and β-intermedin to be highly active in increasing circulating free fatty acids and in causing a release of free fatty acids from depot fat incubated *in vitro*.[2, 3] With this species, too, another extract of the anterior pituitary is effective; this preparation does not share the specific properties of any of the well-recognized pituitary hormones, and, unlike some of the other active agents, it also causes lipemia.[4]

Tests in the rabbit of various pituitary extracts made in our laboratories[5] showed that the crude extract of porcine anterior pituitaries made with hot glacial acetic acid produced fat mobilization, as did the residual solution after removing the corticotropin with oxycellulose.[6] As this solution is usually discarded in the commercial production of corticotropin, it became an abundant source of pituitary material for studies on purification.

Upon neutralizing the solution, some four-fifths of the total solids could be removed in the precipitate without appreciable loss of activity. Studies on the soluble material using the electrophoretic method of Ferguson and Wallace,[7] incorporating a modification of Poulik's[8] discontinuous buffer system and Smithies'[9] starch gel technique, showed it to contain about 50 stainable components which, by this elegant method, appeared as sharp well-separated lines. Assays, using pieces of rabbit fat *in vitro* and measuring the liberation of free fatty acid, showed that eluates of several different segments of the starch exhibited lipolytic activity but the region which was most active was a narrow zone made up of two darkly stained and several faint, closely spaced lines which moved farther toward the anode than any pituitary hormones yet examined. Isolation of the materials giving rise to the two most prominent lines was therefore undertaken. Peptide I, the slower moving component on the starch gel, proved to be the easier to obtain in substantially pure form; peptide II was separated from peptide I and from most extraneous mate-

rial but was not entirely freed of a contaminant. Peptide I was the more active, especially when tested *in vitro*.

Methods.—In a typical procedure, 6 liters of the crude extract in 0.1 *N* acetic acid containing 150 gm of solids was brought to a pH of 6.5 or 7.0 by the addition of about 170 ml of 5 *N* ammonium hydroxide and allowed to stand in the cold overnight. The precipitate was removed on large Buchner funnels with slight suction and washed by percolation with water. The combined filtrate containing about 30 gm of protein-like material was stirred for 2 hr with 100 gm of Dowex 1-X2, 200–400 mesh, which had been freed of the coarsest and finest particles by sedimentation in water and washed in sequence with *N* sodium hydroxide, water, 5 *N* acetic acid, and water. The resin was collected by filtration, washed with water, and poured into a column 3.3 cm in diameter and 19 cm high. Elution with 0.1 *N* acetic acid yielded almost all of the material of interest. Fractions were combined on the basis of the electrophoretic patterns and dried from the frozen state. This procedure yielded 6.3 gm of white powder, 4.2% by weight of the crude extract. This fraction was referred to as an acidic concentrate. A somewhat larger amount of material comprising the most rapidly moving anionic components upon electrophoresis was eluted from the resin by 1 *N* acetic acid, whereas 5 *N* acetic acid removed dark yellow material with an ultraviolet absorption maximum at 260 mμ, presumably nonprotein in nature. Smaller quantities of resin removed the two peptides from the crude extract when adsorption was carried out using a column of resin, as shown in Figure 1.

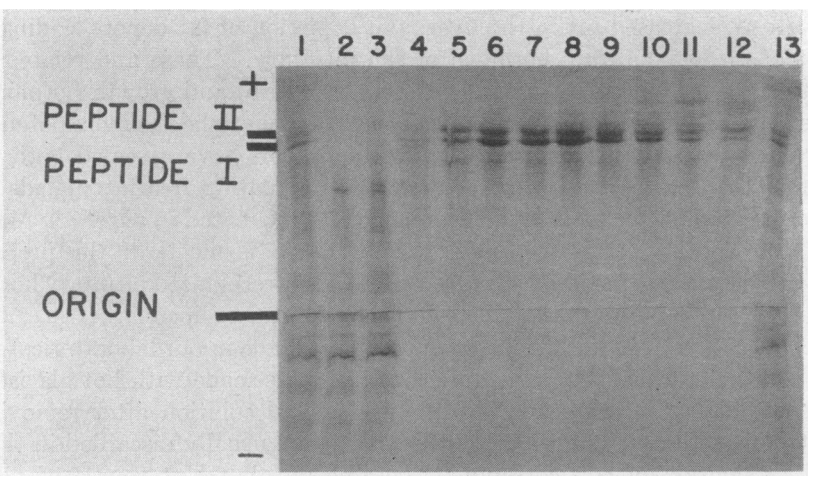

FIG. 1.—Concentration of the 2 peptides using an anion-exchange resin. 10 L of the pH 6.5 soluble crude extract was passed through a column 3.3 × 18 cm of Dowex 1-X2. After washing with ammonium acetate buffer, pH 4.0 and 0.05 *N* in acetic acid, elution was effected with 0.1 *N* acetic acid. Channels 1 and 13, the crude extract; 2 and 3, first and last samples of unadsorbed material showing complete removal of the 2 peptides; 4, buffer eluate; 5 to 12, successive fractions eluted with 0.1 *N* acetic acid. Fractions including those shown in 6 to 8 were combined for further purification.

Chromatographic columns of the same resin, 1.2 cm in diameter and 43 cm high, were used to fractionate 0.5-gm portions of the 0.1 *N* acetic acid eluate of the resin. Successful resolution was achieved by any of several methods; with the resin in the acetate form in each case, prewashing of the column with water permitted all of the desired material to be retained while less acidic substances passed through. Elution with 0.05 or 0.1 *N* acetic acid caused the pH of the effluent solution to fall slowly while a single peak of protein-like substances was eluted (Fig. 2); the maximum of this peak emerged when the pH of the solution was near 3.4. The shape of the peak suggested inhomogeneity, and, indeed, starch gel electrophoresis showed that the first and major portions contained predominantly peptide I, whereas the trailing end of the peak contained this component mixed with peptide II. Much the same elution pattern was seen when a continuous gradient from 0.01 *N* toward 0.1 or 0.2 *N* acetic acid was used or a gradient of ammonium acetate buffer,

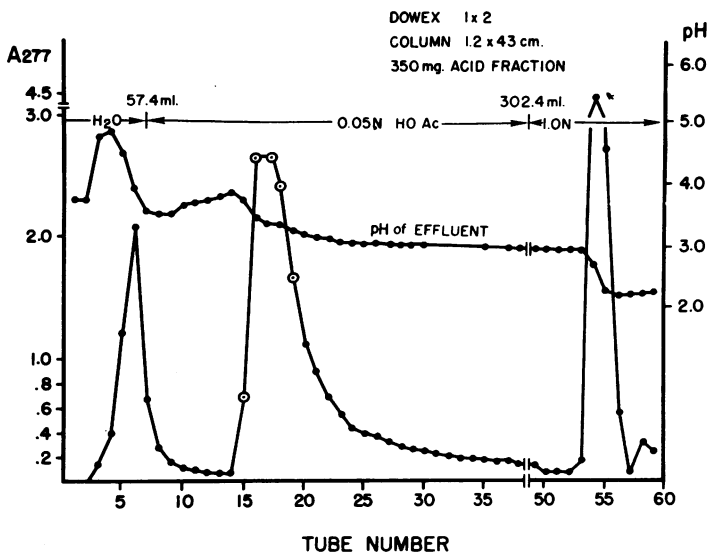

FIG. 2.—Column chromatography of a concentrate of the peptides on an anion-exchange resin. The circled fractions were found by electrophoresis to contain predominantly peptide I; the subsequent fractions eluted by 0.05 *N* acetic acid contained peptide II contaminated with peptide I.

pH 4.0 and 0.1 *N* in acetate was run toward 0.2 *N* acetic acid. The desired substances were not eluted by 0.02 *N* acetic acid or by buffers of ammonium acetate of pH 3.6 to 4.0 when less than 0.02 *N* in acetic acid, but successful chromatography could be carried out in columns equilibrated with ammonium acetate, 0.1 *N* in acetic acid at pH 3.6 and developed with the same buffer. By combining the fractions containing no more than traces of other stainable components after electrophoresis, yields of 115 to 150 mg of peptide I were obtained, or 23 to 30% of the 0.5 gm applied; this represented a yield from the starting material of about 1%, or about 0.15% the weight of the acetone-dried pituitary powder.

To obtain larger amounts of the substances for chemical, biological, and clinical studies, larger batches were processed. Seventy kilograms of whole frozen porcine pituitaries yielded 10.919 kg of acetone-dried anterior lobe powder from which 1.502 kg of crude corticotropin was derived. After corticotropin had been removed by two adsorptions with 120 gm of oxycellulose, the 60 liters of solution were neutralized and the soluble portion stirred with 1,027 gm of resin.[10] A slurry of the resin in water was adjusted with acetic acid to pH 3.68, made into a column 7.6 cm in diameter and 33 cm high, washed with water, and eluted with 0.1 *N* acetic acid. The desired fractions were combined and amounted to 1,500 ml, which upon drying from the frozen state weighed 20.1 gm, a 0.2% yield from the acetone-dried anterior lobe powder. This concentrate was predominantly peptides I and II contaminated by other acidic components; small samples were used for further purification.

Peptides I and II could most easily be separated by taking advantage of the much greater capacity of Dowex 1-X2 for peptide I. Thus, 0.8 gm of the above-mentioned concentrate in 4 ml of water was applied to 4 gm of the resin in a column 0.9 cm in diameter and 8 cm high and the column washed with water. The unadsorbed material, weighing 323 mg, was predominantly II contaminated by impurities and contained no detectable I. Elution with 0.1 *N* acetic acid gave 180 mg of I in a highly concentrated state (Fig. 3).

Separation and purification of peptides I and II could also be achieved using columns of Sephadex G-50 on which peptide I in 0.1 *N* acetic acid was retarded to a greater extent than peptide II (Fig. 4). There was some overlap in the emergence of the two peptides and the most satisfactory separation was obtained when a concentrate of one or the other had first been made with resin. Thus peptide I was isolated in substantially pure form using 0.5 gm of a resin concentrate and a column 1.2 × 54 cm. On combining the fractions shown by electrophoresis to contain only

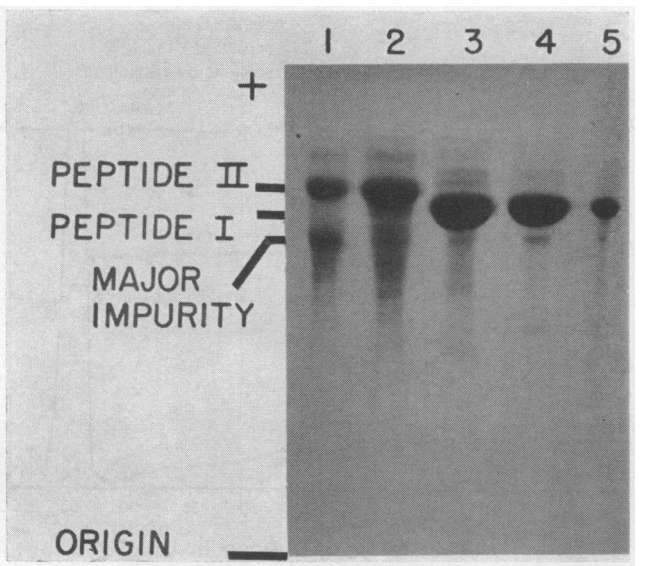

FIG. 3.—Separation of the two peptides by making use of the larger capacity of the resin for peptide I. 0.8 gm of an acidic concentrate applied to 4 gm of Dowex 1-X2 acetate in 4 ml of water, pH 4.0. Channels 1 and 2 show the first and the last fractions of unadsorbed material; channels 3, 4, and 5 are successive eluates with 0.1 *N* acetic acid. The spreading of the 2 main peptides was caused by the large amount of material used to show the minor components.

I and freeze-drying, 166 mg of virtually homogeneous material was recovered. Peptide II could similarly be concentrated but it was not freed of a contaminant with a mobility upon electrophoresis less than that of either active peptide. This impurity is prominently displayed in Figure 3.

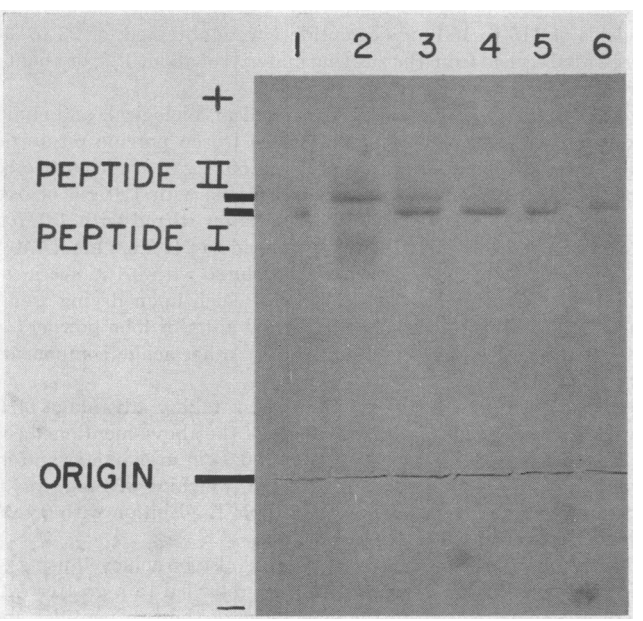

FIG. 4.—Partial separation of the two peptides using Sephadex G-50. 0.5 gm of an acidic concentrate applied to a column 1.2 × 54 cm, solvent 0.1 *N* acetic acid. Channel 1, small amount of unretarded material; 2, peptide II contaminated with a trace of I and a less anionic component; 3, mixture of I and II; 4 to 6, nearly homogeneous I.

Properties.—The seemingly pure peptide I was highly soluble in water, even at the pH of minimal solubility, 4.0 to 4.6, strong solutions being clear and colorless. Upon standing in weak acid, solid gels sometimes formed, a process accelerated by heat. From dilute aqueous solutions, the peptide was precipitated by picric acid, p-hydroxyazobenzene sulfonic acid, and flavianic acid, forming colored amorphous masses or oils. Upon heating at 100° for 4 hr at a pressure of 0.02 mm of mercury, the material which had been dried from the frozen state at 0.02 mm at room temperature lost 11 per cent of its weight. Corrected for this presumed water of hydration, ultraviolet absorption spectra in 0.1 *N* sodium hydroxide[11] suggested 2 moles of tyrosine for 1 of tryptophan and an $A^{1\%}_{1cm}$ of 16 at 290 mμ, indicating a minimal molecular weight of about 5,000.

Electrodiffusion of a concentrate of the acidic components containing the two peptides through cellophane membranes of calibrated porosity by the method of Pierce and Free[12] suggested that peptide I had a molecular weight of less than 10,000; peptide II was estimated to have a molecular weight between 10,000 to 20,000, while the impurity of peptide II was still larger.[13] The ultraviolet absorption spectrum of peptide I exhibited a sharp maximum at 277 mμ and a low minimum at 250 mμ (Fig. 5). The ratio of absorbances 250/277 became smaller with in-

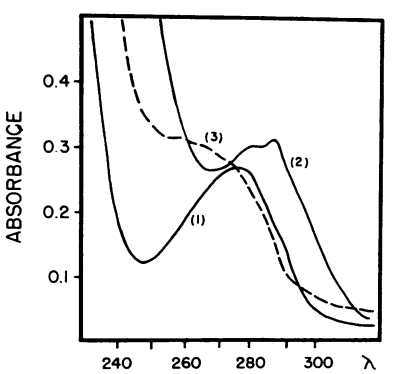

FIG. 5.—Ultraviolet absorption spectra. (1) Peptide I, 0.2 mg/ml in water, pH 4.0; (2) Peptide I, 0.2 mg/ml in 0.1 *N* sodium hydroxide; (3) (dashed line) Peptide II, 1.0 mg/ml in water, pH 4.0.

creasing purification approaching 0.38. This property served as an additional guide to purification. Preparations of peptide II showed no maximum at 277 mμ but a slowly diminishing absorbance from 250 mμ toward longer wavelengths and a shoulder at 270 to 276 (Fig. 5). In 0.1 *N* sodium hydroxide, the spectrum quickly shifted to longer wavelengths and then progressively underwent complex and largely irreversible changes during 8 hr at room temperature.

Tested on isolated pieces of mesenteric or perirenal fat from the rabbit *in vitro*, as little as 0.01 μg of peptide I increased release of free fatty acids; peptide II was very much less active. Injection of 10 μg of peptide I intravenously into rabbits trebled the concentration of circulating free fatty acids in 5 min and doses of 1 mg or more subcutaneously caused lipemia in 12 to 18 hours; peptide II was somewhat less active in these tests.[3] Peptide I like α-intermedin and corticotropin caused an increase in heart rate in the heart-lung preparation of the dog[14] and, in

common with preparations of thyrotropin and α-intermedin, increased the oxygen consumption of isolated leporine adipose tissue incubated *in vitro*.[15]

Summary.—Two metabolically active substances designated peptides I and II have been purified from a crude extract of porcine anterior pituitary glands. Electrophoresis on starch gel served as a guide during fractionation, which was accomplished using an anion-exchange resin and Sephadex G-50. Peptide I of molecular weight near 5,000 was homogeneous upon electrophoresis; peptide II was larger, was not freed of a contaminant, and was less active in causing fat mobilization and lipemia.

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*THE INACTIVATION OF TRYPSIN BY ULTRAVIOLET LIGHT, I. THE CORRELATION OF INACTIVATION WITH THE DISRUPTION OF CONSTITUENT CYSTINE**, [a]

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To elucidate the mechanism(s) of enzymic catalysis requires not only a specification of the amino acid residues involved in the "active center," but also knowledge of their spatial orientation as well as possible rearrangements in intramolecular bonding. Difficulty in finding reagents and/or techniques whose effects are restricted to only a few specific sites in the protein has hampered investigation of these latter factors. The studies reported here indicate that UV (2,537 Å) not only produces restrictive, specific changes in trypsin, but also that under the proper