to give a permanent blue color. During addition of the sodium a precipitate appeared, which dissolved toward the end of the reaction, and the solution became rather yellow. An amount of ammonium iodide equivalent to all the sodium used was added and the ammonia evaporated. The residue was extracted with ether and ethyl acetate, which removed a small amount of yellow impurity, and dissolved in water. The solution was evaporated to dryness *in vacuo* and the residue extracted with 200 ml. of acetone. The insoluble material, which still contained some sodium and ammonium iodide, was triturated with ethanol. This gave 0.65 g. of L-glutamine, which was purified by dissolving it in a small volume of water and adding ethanol; wt. 0.5 g. The product gave a color with Nessler reagent only on standing. This reaction to the test suggested by Archibald[14] indicates the absence of ammonium pyroglutamate in preparations of glutamine. The ninhydrin test was positive and the amino acid showed $[\alpha]^{22}D$ +6.2° (*c* 4.3, water). Fruton[9] gives $[\alpha]^{23}D$ +6.0–6.1° (*c* 3.6, water). For analysis, the material was recrystallized from water–ethanol.

Anal. Calcd. for $C_5H_{10}O_3N_2$: C, 41.0; H, 6.89; N, 19.2. Found: C, 40.9; H, 7.16; N, 18.8.

Paper chromatography showed the glutamine to have an R_f value of 0.54 in phenol:water (3:1) and 0.09 in butanol:water:acetic acid (5:4:1). In both systems the synthetic material gave a single spot and could not be distinguished from an authentic sample of L-glutamine when the two were run either parallel or together. In the butanol:water:acetic acid system, L-glutamine and L-isoglutamine (see subsequent section) had different R_f values.

Tosyl-L-glutamine (24 g.) was dissolved in 700 ml. of liquid ammonia and sodium (7 g.) added[12] to give a permanent blue color. Dowex-50 resin[13] (70 g.) was then added, the ammonia evaporated and the flask thoroughly evacuated. Water (300 ml.) was added and the resin was filtered off and washed with 100 ml. of water. The filtrate was then concentrated to 100 ml. under reduced pressure and diluted with 900 ml. of 95% ethanol. The amino acid was filtered off and washed with ethanol; wt. 11.5 g. A small amount of inorganic material was found to be present and the amino acid was therefore dissolved in 150 ml. of water at 36° and crystallized by the addition of 500 ml. of ethanol; wt. 9 g.

L-Isoglutamine (VIIIa).—The reaction of 1-tosylpyroglutamyl chloride with dry ammonia[5] was carried out as follows. The chloride (14.8 g.) was added in one portion to a saturated solution of ammonia in chloroform (150 ml.). The heat of reaction caused the chloroform to boil. The mixture was then evaporated to dryness, washed with water and filtered. The precipitate of 1-tosylpyroglutamamide weighed 11 g., m.p. 188–190°, and the m.p. was raised to 193–195° by crystallization of the product from 50% ethanol; $[\alpha]^{21}D$ −24.3° (*c* 2.3, acetone). The substance is insoluble in hot chloroform, benzene and ethyl acetate.

Conversion to tosyl-L-isoglutamine and reduction with sodium in liquid ammonia was carried out as described by Harington and Moggridge.[5] When the blue color had been reached, however, Dowex-50 resin[13] (5 g. per g. of tosyl-L-isoglutamine) was added and the L-isoglutamine was isolated according to the procedure described in the preceding section for L-glutamine. The yield of amino acid was 64%; $[\alpha]^{21}D$ +20.5° (*c* 6.1, water). Bergmann and Zervas[7] gave $[\alpha]^{22}D$ +21.1° (*c* 6.5, water). Paper chromatography showed the L-isoglutamine to have an R_f value of 0.52 in phenol:water (3:1) and 0.18 in butanol:water:acetic acid (5:4:1). The isoglutamine melted at 186° dec.

Tosyl-L-glutamide (IX).—This substance was prepared by adding 1-tosylpyroglutamyl chloride to concentrated aqueous ammonia with cooling and then filtering off the product, which was formed in almost quantitative yield.

The diamide could also be prepared in high yield by the action of concentrated ammonium hydroxide on 1-tosylpyroglutamamide (VII) and was obtained from 1-tosylpyroglutamic acid anhydride as mentioned in a preceding section.

Tosyl-L-glutamide melted with decomposition between 210 and 220° depending on the rate of heating, and the m.p. was unaffected by crystallization of the compound from ethanol. The substance is soluble in cold glacial acetic acid, sparingly soluble in ethanol, ethyl acetate, chloroform and acetone; $[\alpha]^{21}D$ +8.3° (*c* 1.7, acetic acid).

Anal. Calcd. for $C_{12}H_{17}O_4N_3S$: C, 48.2; H, 5.72; N, 14.0. Found: C, 48.3; H, 5.89; N, 14.0.

Tosyl-L-glutamide (2 g.) was treated in liquid ammonia (50 ml.) with sodium[12] (0.5 g.) and Dowex-50 resin[13] (5 g.) was added. After evaporation of the ammonia the residue was extracted with hot ethanol and filtered. Evaporation of the filtrate gave a crystalline residue of L-glutamide (IXa) which was characterized by conversion of a small sample to carbobenzoxy-L-glutamide, m.p. 195–196.5°. Fruton[9] reports m.p. 194–196°.

The authors wish to thank Mr. Joseph Albert for carrying out the microanalyses reported herein.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE]

The Synthesis of *p*-Toluenesulfonyl-L-isoleucyl-L-glutaminyl-L-asparagine and Related Peptides[1]

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The synthesis of tosyl-L-isoleucyl-L-glutamine and tosyl-L-isoleucyl-L-glutaminyl-L-asparagine by the coupling of L-glutamine and L-glutaminyl-L-asparagine with tosyl-L-isoleucyl chloride in the presence of aqueous magnesium oxide is described. Two other protected isoleucyl peptides, namely, tosyl-L-isoleucyl-L-leucine and tosyl-L-isoleucylglycine, have also been prepared.

Studies in our laboratory on peptides from the partial hydrolysis of purified oxytocin preparations revealed the sequence isoleucine·glutamic acid·aspartic acid, and in the postulation of a structure for oxytocin which has guided the synthetic approach to the hormone the sequence –isoleucyl·glutaminyl·asparaginyl– was arrived at.[4] In this synthetic program the preparation of L-isoleucyl-L-glutaminyl-L-asparagine with a protective grouping on the amino group of isoleucine was desired. In view of the earlier synthesis of the dipeptide L-glutaminyl-L-asparagine,[5] it was decided to couple this dipeptide with protected L-isoleucine. For this purpose the N-tosyl-(*p*-toluenesulfonyl) derivative was selected and the synthesis of tosyl-L-iso-

(1) A preliminary report of part of this work was made recently [V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis and S. Gordon, THIS JOURNAL, **75**, 4879 (1953)].

(2) Fellow of State Scholarships Foundation of Greece.

(3) Appreciation is expressed to the Lederle Laboratories Division, American Cyanamid Company, for a research grant which has aided greatly in this work.

(4) V. du Vigneaud, C. Ressler and S. Trippett, *J. Biol. Chem.*, **205**, 949 (1953).

(5) J. M. Swan and V. du Vigneaud, THIS JOURNAL, **76**, 3110 (1954)

leucyl-L-glutaminyl-L-asparagine is described herein.

The tosylation of the L-isoleucine was accomplished by a modification of the general method of tosylation which resulted in a yield of over 80%. In order to ascertain whether any racemization had occurred, the tosyl group was removed by reduction with sodium in liquid ammonia according to the method of du Vigneaud and Behrens.[6] The specific rotation of the L-isoleucine so obtained was found to agree with that of an authentic sample of L-isoleucine, which indicated that during tosylation and detosylation processes no appreciable racemization of the isoleucine had occurred.

Treatment of tosyl-L-isoleucine with either phosphorus pentachloride or thionyl chloride gave the corresponding acid chloride as an oil, which was then coupled with L-glutaminyl-L-asparagine with the use of magnesium oxide as the condensing agent[7] to give tosyl-L-isoleucyl-L-glutaminyl-L-asparagine, the desired product, in approximately 50% yield. The compound crystallized as long, thin needles which melted at 223°. Tosyl-L-isoleucyl-L-glutamine was synthesized in a similar manner by the reaction of tosyl-L-isoleucyl chloride with L-glutamine.

Carbobenzoxy-L-isoleucyl-L-glutaminyl-L-asparagine and carbobenzoxy-L-isoleucyl-L-glutamine were also prepared. After cleavage of the tosyl group from the corresponding tosyl peptides the crude reduction products were treated with carbobenzoxy chloride in the presence of magnesium oxide.

The synthesis of two other tosyl peptides, namely, tosyl-L-isoleucyl-L-leucine and tosyl-L-isoleucylglycine, is also reported.

Experimental[8]

Tosyl-L-isoleucine.—The L-isoleucine used in this work was prepared from a commercial mixture of L-isoleucine plus D-alloisoleucine by enzymatic resolution with papain.[9] Tosyl chloride (12 g.) was added to a solution of 6 g. of the L-isoleucine in 40 ml. of 1 *N* NaOH and 20 ml. of water, and the mixture was stirred vigorously at room temperature for 3 hours. The *p*H of the reaction mixture was maintained at approximately 9 by the stepwise addition of 1 *N* NaOH over the reaction period. Unreacted tosyl chloride was filtered off and the filtrate was stirred vigorously and acidified to congo red paper with dilute HCl. The crystalline product was filtered off and washed with water. In several experiments the yield was 10.5–11 g. (80–85%), m.p. 134–135°. After recrystallization from a mixture of ethyl acetate and petroleum ether the m.p. was 135–136°; $[\alpha]^{21}D$ −12.3° (*c* 2, 0.5 *N* $KHCO_3$). McChesney and Swann[10] report m.p. 130–132° (uncor.) for this compound.

Anal. Calcd. for $C_{13}H_{39}O_4NS$: C, 54.7; H, 6.66; N, 4.91. Found: C, 54.4; H, 6.86; N, 4.90.

The tosyl group was removed by dissolving 1.5 g. of the tosyl-L-isoleucine in 50 ml. of liquid ammonia and adding sodium in portions until a permanent blue color was obtained (approximately 0.6 g.). The solution was then decolorized by addition of the ion-exchange resin, Dowex-50.[5] After evaporation of the ammonia the residue was dissolved in 40 ml. of water, the *p*H adjusted to 6 with acetic acid and the solution decolorized with Norit-A and concentrated to a small volume *in vacuo*. On cooling the solution L-isoleucine, $[\alpha]^{21}D$ +40.6° (*c* 5, 6 *N* HCl), separated.

Tosyl-L-isoleucyl-L-glutamine.—A mixture of 4.2 g. of tosyl-L-isoleucine and 20 ml. of thionyl chloride was heated on a water-bath (45°) under anhydrous conditions for 25 minutes and the excess thionyl chloride was removed by evaporation first at the water pump and then at the oil pump. The product was obtained as a pale yellow oil (4.3 g.).

One-fourth of the crude acid chloride was dissolved in 5 ml. of anhydrous benzene and added in portions with shaking over a period of 1 hour to an ice-cold suspension of 0.8 g. of L-glutamine and 0.2 g. of magnesium oxide in 8 ml. of water. If the *p*H dropped below 8 during this time additional magnesium oxide was added. The reaction mixture was shaken for another 15 minutes, diluted with 25 ml. of water and filtered. The filtrate was extracted with ether and the aqueous phase acidified to *p*H 2 with dilute HCl. The crude product was filtered off and washed with water; wt. 0.47 g., m.p. 167–170°. Crystallization from ethanol gave 0.30 g. of the tosyl dipeptide, m.p. 203–204°, $[\alpha]^{21}D$ −3.1° (*c* 1.1, 0.5 *N* $KHCO_3$).

Anal. Calcd. for $C_{18}H_{27}O_6N_3S$: C, 52.3; H, 6.58; N, 10.2. Found: C, 51.8; H, 6.62; N, 10.0.

Carbobenzoxy-L-isoleucyl-L-glutamine.—Tosyl-L-isoleucyl-L-glutamine (0.4 g.) was dissolved in 50 ml. of liquid ammonia and sodium was added until a permanent blue color was obtained (approximately 0.1 g.). Ammonium chloride was added equivalent to the sodium and the ammonia was evaporated, the last traces being removed *in vacuo*. The residue was dissolved in 8 ml. of water and the *p*H of the solution brought to 4 with HCl. After two extractions with ether the aqueous phase was cooled in an ice-water bath and 0.1 g. of magnesium oxide added. Carbobenzoxy chloride (0.28 g.) was added in three portions over a period of 15 minutes with vigorous shaking. After an additional 20 minutes the mixture was acidified to congo red paper with 5% HCl. The resulting white solid was filtered off and washed with water; wt. 0.3 g. After crystallization and recrystallization from ethanol 0.25 g. (64%) was obtained, m.p. 184–185°, $[\alpha]^{21}D$ −18.8° (*c* 1.3, 0.5 *N* $KHCO_3$).

Anal. Calcd. for $C_{18}H_{27}O_6N_3$: C, 58.0; H, 6.91; N, 10.7. Found: C, 57.9; H, 6.89; N, 10.7.

Tosyl-L-isoleucyl-L-glutaminyl-L-asparagine.—Tosyl-L-isoleucine (4.2 g.) was dissolved in 20 ml. of anhydrous ether, 3.2 g. of phosphorus pentachloride was added and the mixture was shaken until almost all of the phosphorus pentachloride had dissolved. The remainder was filtered off and the filtrate was evaporated at the water pump followed by at least 45 minutes at the oil pump to give a colorless oil (4.3 g.).

The latter was dissolved in 22 ml. of anhydrous dioxane and added over a period of 1 hour with vigorous shaking to an ice-cold suspension of 3.6 g. of L-glutaminyl-L-asparagine and 1.25 g. of magnesium oxide in 35 ml. of water. Care was taken to keep the *p*H of the mixture above 8 as in the preparation of the tosyl-L-isoleucyl-L-glutamine. After addition of the chloride the mixture became almost solid due to the precipitation of the magnesium salt of the tosyl tripeptide. Water (20 ml.) was added and the magnesium salt was separated by filtration. A suspension of this salt in 500 ml. of cold water was brought to *p*H 3 by addition of 5% HCl and the resulting precipitate was filtered off and washed well with water; wt. (in several experiments) 3.3–3.5 g. Acidification of the filtrate from the magnesium salt of the tosyl tripeptide gave an additional 0.9–1 g. The yield of crude product, m.p. 219–220°, was 4.2–4.5 g. A portion was purified by precipitation from dilute aqueous $KHCO_3$ with HCl. The tosyl tripeptide crystallized in the form of long, thin needles, m.p. 223°, $[\alpha]^{21}D$ −30.7° (*c* 1.8, 0.5 *N* $KHCO_3$).

Anal. Calcd. for $C_{22}H_{33}O_8N_5S$: C, 50.1; H, 6.30; N, 13.3. Found: C, 49.8; H, 6.51; N, 13.3.

Carbobenzoxy-L-isoleucyl-L-glutaminyl-L-asparagine.—This compound was prepared from 0.5 g. of tosyl-L-isoleucyl-L-glutaminyl-L-asparagine according to the procedure already described for the synthesis of carbobenzoxy-L-isoleucyl-L-glutamine. The crude product was purified by precipitation from dilute aqueous $KHCO_3$ (which had been extracted twice with ether to remove carbobenzoxy chloride) with HCl; yield 0.25 g. (52%), m.p. 203–204°, $[\alpha]^{21}D$ −35.5° (*c* 1, 0.5 *N* $KHCO_3$).

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(7) M. Bergmann and L. Zervas, *Ber.*, **65B**, 1192 (1932).

(8) Capillary melting points were determined for all compounds and are corrected.

(9) D. G. Doherty and E. A. Popenoe, Jr., *J. Biol. Chem.*, **189**, 447 (1951).

(10) E. W. McChesney and W. K. Swann, Jr., THIS JOURNAL, **59**, 1116 (1937).

Anal. Calcd. for $C_{23}H_{33}O_8N_5$: C, 54.4; H, 6.58; N, 13.8. Found: C, 54.3; H, 6.66; N, 13.4.

Ethyl Tosyl-L-isoleucylglycinate.—A solution of tosyl-L-isoleucyl chloride (prepared from 2.9 g. of tosyl-L-isoleucine and phosphorus pentachloride) in 15 ml. of anhydrous ether was added slowly to a suspension of 2.5 g. of ethyl glycinate hydrochloride and 3.5 ml. of triethylamine in 50 ml. of anhydrous ether and the mixture was allowed to stand at room temperature for 12 hours. The white precipitate was filtered off and washed with ether. After trituration with water to remove the triethylamine hydrochloride, 3.34 g. of product, m.p. 159–160°, was obtained; yield 89%. Another 0.25 g., m.p. 152–154°, was afforded by extraction of the ether filtrate successively with water, dilute HCl, dilute aqueous $KHCO_3$ and water, followed by removal of the ether *in vacuo*. Recrystallization of the second crop from ethanol raised the m.p. to 158–160°. For analysis, the tosyl dipeptide ester was recrystallized twice from ethanol and then melted at 160–161°.

Anal. Calcd. for $C_{17}H_{26}O_5N_2S$: C, 55.1; H, 7.07; N, 7.56. Found: C, 55.1; H, 7.30; N, 7.55.

Tosyl-L-isoleucylglycine.—The tosyl dipeptide ester (1.6 g.) was dissolved in 9 ml. of ethanol and 5 ml. of 1 *N* NaOH was added. The mixture was warmed on a water-bath (70°) for 1 minute, allowed to stand at room temperature for 2 hours, acidified to congo red paper with HCl and then concentrated *in vacuo* to dryness. The residue was dissolved in ethyl acetate plus a few drops of water and this solution was extracted twice with dilute aqueous $KHCO_3$. Acidification of the aqueous phase to congo red paper precipitated a crystalline product which was filtered off and washed with water; wt. 1 g. (71%), m.p. 185°. The m.p. remained unchanged after precipitation of the tosyl dipeptide from dilute aqueous $KHCO_3$ with HCl; $[\alpha]^{21}D$ −26.7° (*c* 1.1, 0.5 *N* $KHCO_3$).

Anal. Calcd. for $C_{15}H_{22}O_5N_2S$: C, 52.6; H, 6.47; N, 8.18. Found: C, 52.3; H, 6.63; N, 8.20.

Unchanged ethyl ester could be recovered by concentration of the ethyl acetate phase *in vacuo*.

Methyl Tosyl-L-isoleucyl-L-leucinate.—Tosyl-L-isoleucyl chloride (from 4.2 g. of tosyl-L-isoleucine and phosphorus pentachloride) was dissolved in 20 ml. of anhydrous ether and added to a suspension of 3.6 g. of methyl L-leucinate hydrochloride and 5 ml. of triethylamine in approximately 75 ml. of anhydrous ether. After 12 hours the precipitate was filtered off, washed with ether and triturated with water; wt. 4.6 g., m.p. 145–146°. The ether filtrate was shaken successively with water, dilute HCl, dilute aqueous $KHCO_3$ and water. Removal of the ether *in vacuo* and recrystallization of the residue from ethanol gave an additional 0.5 g. of product, m.p. 146–147°; over-all yield 85%. After two recrystallizations from ethanol, the m.p. was 147–148°.

Anal. Calcd. for $C_{20}H_{32}O_5N_2S$: C, 58.2; H, 7.82; N, 6.79. Found: C, 58.2; H, 7.84; N, 6.87.

Tosyl-L-isoleucyl-L-leucine.—This tosyl dipeptide was prepared by saponification of 2 g. of methyl tosyl-L-isoleucyl-L-leucinate (dissolved in 20 ml. of ethanol) with 5.5 ml. of 1 *N* NaOH according to the procedure described for the preparation of tosyl-L-isoleucylglycine; wt. 1 g., m.p. 152–154°. The ethyl acetate phase containing unreacted ester was concentrated *in vacuo* and the residual oil was dissolved in 8 ml. of ethanol and saponified in the same manner using 2 ml. of 1 *N* NaOH. The second crop (0.3 g.) melted at 151–153°. For analysis, a portion was recrystallized from a mixture of ethyl acetate and petroleum ether and dried; m.p. 163–165°; $[\alpha]^{21}D$ −24.7° (*c* 1.1, 0.5 *N* $KHCO_3$).

Anal. Calcd. for $C_{19}H_{30}O_5N_2S$: C, 57.3; H, 7.58. Found: C, 57.2; H, 7.73.

The authors wish to thank Mr. Joseph Albert for carrying out the microanalyses reported herein.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE]

The Synthesis of Oxytocin[1]

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A cyclic octapeptide amide (I) having the hormonal activity of oxytocin has been synthesized through the condensation of N-carbobenzoxy-S-benzyl-L-cysteinyl-L-tyrosine and the heptapeptide amide L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (IVa) to yield the protected nonapeptide amide VI followed by reduction with sodium in liquid ammonia and oxidation of the resulting sulfhydryl nonapeptide. IVa was prepared by the condensation of S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide with tosyl-L-isoleucyl-L-glutaminyl-L-asparagine followed by removal of the tosyl group from the condensation product. The biologically active synthetic material thus obtained has been purified by countercurrent distribution and compared with natural oxytocin as to potency, specific rotation, partition coefficients, amino acid composition, electrophoretic mobility, infrared pattern, molecular weight, enzymatic and acid inactivation and chromatography on the resin IRC-50. The synthetic material and natural oxytocin were also compared with respect to milk ejection and induction of labor in the human as well as rat uterus contraction *in vitro*. The crystalline flavianates prepared from the synthetic material and from natural oxytocin were found to have the same crystalline form, melting point and mixed melting point. All of these comparisons afforded convincing evidence of the identity of the synthetic product with natural oxytocin. This synthesis thus constitutes the first synthesis of a polypeptide hormone.

Oxytocin, the principal uterine-contracting and milk-ejecting hormone of the posterior pituitary gland,[5] was obtained from the latter in this Laboratory in highly purified form,[6–8] and isolated as a crystalline flavianate.[8] The purification was effected by application of countercurrent distribution[9,10] to posterior pituitary material which

(1) A preliminary report of this work has appeared [V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis and S. Gordon, THIS JOURNAL, **75**, 4879 (1953)].

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(3) Fulbright Scholar on Smith-Mundt grant-in-aid, on leave from Wool Textile Research Laboratory, C.S.I.R.O., Australia.

(4) Fellow of State Scholarships Foundation of Greece.

(5) The uterine-contracting activity of pituitary extracts was reported by H. H. Dale in 1906 [*J. Physiol.*, **34**, 163], and the milk-ejecting activity by I. Ott and J. C. Scott in 1910 [*Proc. Soc. Exp. Biol. Med.*, **8**, 48]. For a discussion of numerous chemical and biological investigations of the posterior pituitary gland including the early work on the subject, reference might be made to the review by H. Waring and F. W. Landgrebe ["The Hormones," Vol. 2, G. Pincus and K. V. Thimann, Ed., Academic Press, Inc., New York, N. Y., 1950, pp. 427–514].

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