acid than could be produced by the utilization of riboflavin alone in tubes containing from 2.5–35 μg of the analog in addition to the 0.30 μg of riboflavin, is due to the appearance and culturing of a mutant form of *L. casei* which is able to utilize the analog for its flavin requirements.[24]

B. For the Rat.—Weanling male rats of the Wistar strain[25] were used. The conditions under which the animals were maintained and the riboflavin-deficient diet used have been described before.[26] When the animals became satisfactorily riboflavin deficient,[26] the flavin supplements were administered by stomach tube as a solution or suspension in 0.5 ml of 6% gum acacia solution each day immediately before they were fed. The deficient control animals were given the corresponding vehicle without the flavin. All tests were continued for 28 days from the time the animals became deficient.

Results

Table I shows that the administration of 5 μg/day of 7-bromo-8-methylflavin results in a stimulation of growth (group 2). The administration of 25 μg/day (group 4) results in growth which is approximately equal to that shown by the animals receiving 10 μg/day of riboflavin (group 3). All animals survived the administration of 50 μg/day (group 6). This is to be compared with the survival of only 40% of animals given 50 μg/day of the 7-chloro-8-methylflavin.[10] At 250 μg/day, the lethal property of the analog is revealed by death of one-third of the animals receiving it (group 7). That the compound is lethal is unequivocally demonstrated by the administration of 500 μg/day (group 8), which results in the death of two-thirds of the animals. Since the lethality of the analog is reversed by the simultaneous administration of riboflavin (group 10), it may be concluded that the analog is functioning as a reversible antagonist of riboflavin in the rat. The 40 μg/day of riboflavin does not completely reverse the antagonistic action of 500 μg/day of the analog. The ratio of analog to riboflavin of 5:1 is less disadvantageous to the animal than a ratio of 12:1 (group 10). The additive effect of the two flavins when small quantities are administered is shown by the growth response to a mixture of 50 μg of the analog and 10 μg of riboflavin/day.

TABLE I

GROWTH OF RATS RECEIVING ANALOG, RIBOFLAVIN, OR ANALOG PLUS RIBOFLAVIN

Group	Daily supplement	Wt gain, g[a]	Survivors
1	H_2O	5 ± 2	8/8
2	5 μg of analog	16 ± 2	8/8
3	10 μg of riboflavin	55 ± 4	8/8
4	25 μg of analog	62 ± 3	9/9
5	15 μg of riboflavin	76 ± 2	7/7
6	50 μg of analog	89 ± 3	9/9
7	250 μg of analog	103 ± 6	6/9
8	500 μg of analog	...[b]	3/9
9	50 μg of analog + 10 μg of riboflavin	117 ± 7	8/8
10	500 μg of analog + 40 μg of riboflavin	105 ± 5	8/8

[a] Net weight gain of survivors for the 4-week test period plus or minus an estimate of the standard error of the mean. If these 28-day means are plotted and a line drawn to the origin, an excellent reproduction of the plot of the average rate of growth of the various groups is obtained. [b] These animals showed the same rate of growth for the first 15 days (average weight of group 8 = 63 g) as shown by the animals in group 10 (average weight = 65 g), although two animals had already died. No weight is given because two-thirds of the animals had died. The three remaining animals showed a net weight gain by the 28th day of 63, 90, and 105 g.

(24) For further details of this type of occurrence, see ref 10.
(25) CFN rats, Carworth Farms, New City, N. Y.
(26) J. P. Lambooy and H. V. Aposhian, *J. Nutr.*, **47**, 539 (1952).

Synthesis and Pharmacological Activity of 4-D-Glutamine-oxytocin,[1] 5-D-Asparagine-oxytocin, and 4-D-Glutamine-5-D-asparagine-oxytocin

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5-D-Asparagine- and 4-D-glutamine-5-D-asparagine-oxytocin have been synthesized. Pharmacological testing showed that 4-D-glutamine- and 5-D-asparagine-oxytocin possess very low specific oxytocic and vasodepressor activities, and 4-D-glutamine-5-D-asparagine-oxytocin had no activity in these tests; however, by cumulative dose–response studies for oxytocic activity, it was found that 4-D-glutamine- and 5-D-asparagine-oxytocin had similar "intrinsic" activity to oxytocin

With a view to study the structure–activity relationship of oxytocin and related peptides, the synthesis of 5-D-asparagine- and 4-D-glutamine-5-D-asparagine-oxytocin and the pharmacological testing of these and 4-D-glutamine-oxytocin, synthesized earlier,[1] has been carried out.

p-Nitrophenyl benzyloxycarbonyl-D-asparaginate (I) was prepared from benzyloxycarbonyl-D-asparagine and *p*-nitrophenol by treatment with dicyclohexylcarbodiimide. I on condensation with S-benzylcysteinylprolylleucylglycinamide[2] yielded benzyloxycarbonyl-D-asparaginyl-S-benzylcysteinylprolylleucylglycinamide (II). II on treatment with HBr–AcOH followed by condensation with *p*-nitrophenyl benzyloxycarbonylglutaminate gave benzyloxycarbonylglutaminyl-

(1) A. S. Dutta and N. Anand, *Indian J. Chem.*, **3**, 232 (1965).
(2) M. Bodanzsky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

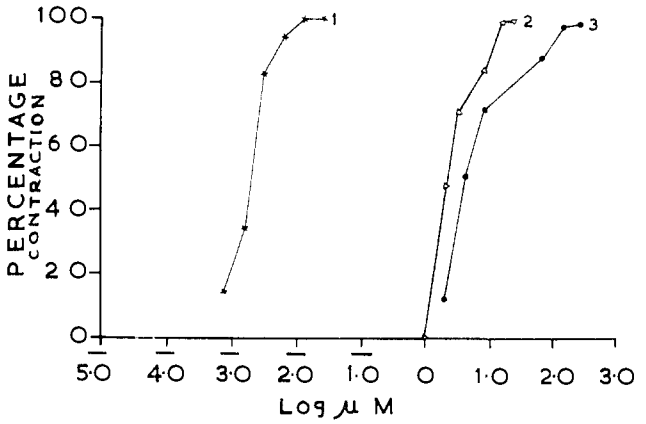

Figure 1.—Log dose–response curves for oxytocin and analogs by cumulative dose procedure: 1, oxytocin; 2, 5-D-asparagine-oxytocin; 3, 4-D-glutamine-oxytocin.

D-asparaginyl-S-benzylcysteinylprolylleucylglycinamide (III). Debenzyloxycarbonylation of III in a similar manner followed by condensation with N-benzyloxycarbonyl-S-benzylcysteinyltyrosylisoleucyl azide[3] gave N-benzyloxycarbonyl-S-benzylcysteinyltyrosylisoleucylglutaminyl-D-asparaginyl-S-benzylcysteinylprolylleucylglycinamide (IV). Compound IV on treatment with sodium and liquid ammonia followed by oxidation and purification by countercurrent distribution[4] (200 cycles) gave 5-D-asparagine-oxytocin.

p-Nitrophenyl benzyloxycarbonyl-D-glutaminate (V) was condensed with D-asparaginyl-S-benzylcysteinylprolylleucylglycinamide to give benzyloxycarbonyl-D-glutaminyl-D-asparaginyl-S-benzylcysteinylprolylleucylglycinamide (VI), which on HBr–AcOH treatment followed by condensation with N-benzyloxycarbonyl-S-benzylcysteinyltyrosylisoleucyl azide yielded N-benzyloxycarbonyl-S-benzylcysteinyltyrosylisoleucyl-D-glutaminyl-D-asparaginyl-S-benzylcysteinylprolylleucylglycinamide (VII). This with sodium and liquid ammonia followed by oxidation and purification as described above yielded 4-D-glutamine-5-D-asparagine-oxytocin.

Biological Activities.—The oxytocic and the vasodepressor activities of these analogs were studied and compared with those of synthetic oxytocin (Syntocinon).

Effect on the Uterus.—The oxytocic activity was determined on the isolated rat uterus of animals, pretreated with estrogen, both by the four-point assay method[5] and by the cumulative dose–response procedure[6a] as used by Rudinger and Krajci.[6b] The antagonism of the analogs to the prototype was studied by administration of the analogs along with the prototype, at a dose level of the latter which would cause half-maximal contraction. The specific activity of these analogs in international units is given in Table I, while a plot of the cumulative dose–response curves is given in Figure 1. In the case of D-glutamine-oxytocin and D-asparagine-oxytocin the dose–response curves run parallel to those of oxytocin, displaced toward higher concentration, and at sufficiently high dose levels, these analogs gave the same maximal effect as oxytocin. This shows that these analogs which have weak specific activity have similar intrinsic activity as the prototype, but have much lower affinity, while D-glutamine-D-asparagine-oxytocin differs from oxytocin in having reduced intrinsic activity as well as reduced affinity. None of these analogs antagonized the activity of oxytocin; indeed D-glutamine- and D-asparagine-oxytocin potentiated the action of oxytocin. These results would seem to indicate that the L-glutamine and L-asparagine residues are not critical for producing the pharmacological response to oxytocin, but are more concerned with the affinity of the molecule. The reduced binding of an antipode to the receptor for the prototype due to changes in stereochemistry of the molecule is understandable.

TABLE I

Analog	Specific activity, IU/mg: Oxytocic effect (isolated rat uterus)	Specific activity, IU/mg: Blood pressure effect (fowl)
5-D-Asparagine-oxytocin	0.2	0.36
4-D-Glutamine-oxytocin	0.1	1.0
4-D-Glutamine-5-D-asparagine-oxytocin	...	...

Effect on the Blood Pressure of Fowl.—White Leghorn cockrels weighing approximately 1 kg were used in these experiments.[5] Doses of the test substance and standard substance were given alternately until they produced equal effect and this was repeated in three dose levels. The value expressed in international units per milligram was calculated from the mean of three observations. A study of antagonism was made by injecting an ineffective dose of test substance in combustion with an effective dose of standard substance and observing any difference from that induced by an effective dose of standard substance. The results are summarized in Table I. None of the analogs showed any antagonism to Syntocinon.

Experimental Section

Unless otherwise stated all the amino acids are of L configuration. Paper chromatographic analyses were carried out by the descending method using one of the following solvents: (A) 1-butanol–acetic acid–water (4:1:1), (B) 1-butanol–acetic acid–water (4:1:5), and (C) *m*-cresol–phenol (1:1) pH 9.3 and borate buffer. The amino acids present in all the analogs were estimated by hydrolyzing with 6 *N* HCl at 100–110° for 20 hr in sealed tubes, followed by two-dimensional chromatography on Whatman No. 3 MM filter paper sheets using A and C as developing solvents. The papers were sprayed with ninhydrin solution (0.4% in acetone). The spots were eluted with 75% ethanol and the color intensity was measured at 540 mμ; the concentration was read from a standard curve drawn for each amino acid separately. The color intensity for proline was measured at 420 mμ. All melting points are uncorrected.

5-D-Asparagine-oxytocin. *p*-Nitrophenyl Benzyloxycarbonyl-D-asparaginate (I).—Dicyclohexylcarbodiimide (3.9 g) was added to a solution of benzyloxycarbonyl-D-asparagine (5 g) and *p*-nitrophenol (3.2 g) in dimethylformamide, and the solution was stirred for 1 hr at 0° and for 1 hr at 25°. The product was isolated in a similar manner as described for V; yield 3 g (41%), mp 168°, $[\alpha]^{31}D$ +30° (c 2, dimethylformamide).

Anal. Calcd for $C_{18}H_{17}N_3O_7$: C, 55.79; H, 4.42; N, 10.82. Found: C, 55.85; H, 4.60; N, 10.74.

Benzyloxycarbonyl-D-asparaginyl-S-benzylcysteinylpropylleucylglycinamide (II).—A mixture of I (2.5 g) and S-benzylcysteinylpropylleucylglycinamide (3 g) in ethyl acetate (50 ml)

(3) R. A. Boissonnas, S. Guttmann, P. A. Jaquenoud, and J. P. Waller, *Helv. Chim. Acta*, **38**, 1491 (1955).

(4) P. A. Jaquenoud and R. A. Boissonnas, *ibid.*, **42**, 788 (1959).

(5) J. H. Burn, D. J. Finney, and L. G. Goodwin, "Biological Standardisation," Oxford University Press, London, 1950, p 177.

(6) (a) E. J. Ariens and W. M. De Grunt, *Arch. Intern. Pharmacodyn.*, **99**, 193 (1954); (b) J. Rudinger and I. Krajci, *Experientia*, **18**, 585 (1962).

was stirred at room temperature for 48 hr. The precipitate which separated was filtered, washed with ethyl acetate and ethanol, and dried; yield 3.5 g (77%), mp 230°, $[\alpha]^{20}D$ −45° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{35}H_{47}N_7O_8S$: C, 57.9; H, 6.53; N, 13.5. Found: C, 58.20; H, 6.62; N, 13.57.

Benzyloxycarbonylglutaminyl-D-asparaginyl-S-benzylcysteinylpropylleucylglycinamide (III).—II (1.5 g) was dissolved in glacial acetic acid (15 ml), and 4 *N* HBr in acetic acid (15 ml) was added and kept for 1 hr at 20°. Excess of dry ether was then added and the precipitate was filtered, washed with ether, and dried (NaOH) *in vacuo*. The hydrobromide was dissolved in dimethylformamide (10 ml), triethylamine (1 ml) was added, followed by *p*-nitrophenyl benzyloxycarbonylglutaminate (0.9 g). The solution was stirred at room temperature for 24 hr and diluted with ethyl acetate (150 ml), and the precipitate was filtered and washed with ethyl acetate, and alcohol; yield 1.5 g (85%), mp 238°, $[\alpha]^{20}D$ −43° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{40}H_{55}N_9O_{10}S$: C, 56.25; H, 6.49; N, 14.8. Found: C, 56.39; H, 7.02; N, 14.73.

N-Benzyloxycarbonyl-S-benzylcysteinyltyrosylisoleucylglutaminyl-D-asparaginyl-S-benzylcysteinylprolylleucylglycinamide (IV).—A solution of N-benzyloxycarbonyl-S-benzylcysteinyltyrosylisoleucyl hydrazide (0.65 g) in dimethylformamide (10 ml) was cooled to −5°, and 4 *N* HCl (1.5 ml) and dimethylformamide (6 ml) were added at −5°, followed by 5 *M* $NaNO_2$ (0.2 ml). The solution was stirred for 5 min at −5°. Triethylamine (0.85 ml) in ethyl acetate (30 ml) was then added, triethylamine hydrochloride was filtered, and the filtrate was dried (Na_2SO_4). L-Glutaminyl-D-asparaginyl-S-benzylcysteinylprolylleucylglycinamide (1.1 g) obtained by the HBr in acetic acid treatment of III in dimethylformamide (10 ml) was added, and the solution was stirred for 3 days at 20°. Ethyl acetate (100 ml) was then added, and the precipitate was filtered and washed with ethyl acetate and hot methanol; yield 0.47 g (34%), mp 245°, $[\alpha]^{20}D$ −35° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{65}H_{86}N_{12}O_{14}S_2$: C, 58.91; H, 6.49; N, 12.68. Found: C, 58.61; H, 6.68; N, 12.98.

Compound IV (0.4 g) was dissolved in dry liquid NH_3 (120 ml) and sodium was added in small amounts until a permanent blue color persisted for 15 min. Ammonium chloride was then added until the white precipitate formed during the reaction dissolved and the solution became colorless. Ammonia was removed *in vacuo*, the residue was dissolved in water (200 ml), the pH of the solution was adjusted to 6.5, and CO_2-free air was bubbled through the solution until the sodium nitroprusside test for SH groups became negative (*ca.* 4 hr). The solution was freeze dried, and the crude material was purified by countercurrent distribution (200 transfers). The fractions were analyzed by ultraviolet spectroscopy (275 mμ) and Folin's estimations. Fractions 35–70 (K = 0.33) were mixed and evaporated to give pure 5-D-asparagine-oxytocin. The pure peptide gave a single spot on paper chromatographic analysis in solvent A, R_f 0.48. Amino acids were found to be present in the following ratio: glycine 1, leucine 1, proline 0.94, cysteine 1.88, asparatic acid 1.01, glutamic acid 0.96, isoleucine 1.03, and tyrosine 1.10.

4-D-Glutamine-5-D-asparagine-oxytocin. *p*-Nitrophenyl Benzyloxycarbonyl-D-glutaminate (V).—Dicyclohexylcarbodiimide (5.15 g) was added to a solution of benzyloxycarbonyl-D-glutamine (5 g) and *p*-nitrophenol (3.2 g) in dimethylformamide cooled to 0° and the solution was stirred for 1 hr at 0° and for 1 hr at 25°. The N,N′-dicyclohexylurea was filtered, the filtrate was diluted with water, and the product filtered and crystallized from ethanol; yield 4 g (40%), mp 155°, $[\alpha]^{31}D$ +24° (*c* 2, dimethylformamide).

Anal. Calcd for $C_{19}H_{19}N_3O_7$: C, 56.85; H, 4.77; N, 10.5. Found: C, 56.70; H, 4.8; N, 10.62.

Benzyloxycarbonyl-D-glutaminyl-D-asparaginyl-S-benzylcysteinylprolylleucylglycinamide (VI).—D-Asparaginyl-S-benzylcysteinylprolylleucylglycinamide (2 g) was dissolved in dimethylformamide (10 ml) and condensed with *p*-nitrophenyl benzyloxycarbonyl-D-glutaminate (1.35 g). After 24 hr ethyl acetate (100 ml) was added, and the hexapeptide was filtered and washed with ethyl acetate and ethanol; yield 2.2 g (78%), mp 244°, $[\alpha]^{31}D$ −45° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{10}H_{55}N_9O_{10}S$: C, 56.25; H, 6.49; N, 14.8. Found: C, 55.99; H, 6.72; N, 14.66.

N-Benzyloxycarbonyl-S-benzylcysteinyltyrosylisoleucyl-D-glutaminyl-D-asparaginyl-S-benzylcysteinylprolylleucyl lycinamide (VII).—To a cooled solution of N-benzyloxycarbonyl-S-benzylcysteinyltyrosylisoleucyl hydrazide (1.34 g) in dimethylformamide (15 ml) were added 4 *N* HCl (3 ml) and dimethylformamide (20 ml) followed by 5 *M* $NaNO_2$ (0.4 ml). The azide was worked up in the usual manner and condensed with D-glutaminyl-D-asparaginyl-S-benzylcysteinylprolylleucylglycinamide (VIII, 2 g). VIII was prepared from VI in an identical manner as described for III. After 3 days the product was filtered after adding ethyl acetate to the suspension and washed with ethyl acetate and hot methanol; yield 0.82 g (22%), mp 245°, $[\alpha]^{31}D$ −30° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{65}H_{86}N_{12}O_{14}S$: C, 58.91; H, 6.49; N, 12.68. Found: C, 58.71; H, 6.8; N, 12.89.

Compound VII (0.6 g) was reduced with sodium–liquid ammonia as described above for 5-D-asparagine-oxytocin and then oxidized at pH 6.5 with CO_2-free air for 4 hr. The crude 4-D-glutamine-5-D-asparagine-oxytocin thus obtained was purified by countercurrent distribution. After 424 transfers fractions 15–36 (K = 0.06) containing pure 4-D-glutamine-5-D-asparagine-oxytocin were pooled and evaporated to dryness. It gave a single spot on paper chromatography in solvent A, R_f 0.34. Amino acids were found to be present in the following ratio: glycine 1, leucine 1.05, proline 0.96, cysteine 1.90, aspartic acid 1.10, glutamic acid 0.98, isoleucine 0.98, and tyrosine 1.11.

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