Table I. Numerical Relationships within the Fibonacci Search

No. of internal points	Points which correspond to the analogs to be made first	Total no. of analogs required to locate max
2	1 and 2	2
4	2 and 3	3
7	3 and 5	4
12	5 and 8	5
20	8 and 13	6
33	13 and 21	7

Table II. Data of Cutler, *et al.*, of compounds **1** as reported by Hansch and Clayton

No.	R	pC[a]	No.	R	pC[a]
0	C_7		7	C_{14}	5.12
1	C_8	3.00	8	C_{15}	5.14
2	C_9	3.02	9	C_{16}	5.16
3	C_{10}	3.57	10	C_{17}	4.70
4	C_{11}	4.06	11	C_{18}	4.71
5	C_{12}	4.61	12	C_{19}	4.73
6	C_{13}	5.10	13	C_{20}	

[a] pC is the negative logarithm of the MIC against *Clostridium welchii*.

Table III. Fibonacci Search for the Data in Table II

Point no.	Activity	Point no.	Activity	Limits
5 (C_{12})	4.61	8 (C_{15})	5.14	$C_{12} \leq$ max $\leq C_{20}$
8 (C_{15})	5.14	10 (C_{17})	4.70	$C_{12} \leq$ max $\leq C_{17}$
7 (C_{14})	5.12	8 (C_{15})	5.14	$C_{14} \leq$ max $\leq C_{17}$
8 (C_{15})	5.14	9 (C_{16})	5.16	$C_{15} \leq$ max $\leq C_{17}$

chosen, only one additional point is required in each successive step.

Observe that there were 12 analogs in this series but that the optimal one could be found by making only five analogs. Moreover, most of the compounds indicated lie in the neighborhood of the maximum. None of the other compounds in the series were required to locate the maximum.

The search can actually extend over as large a chain length as desirable, which may be practically determined by the number of compounds which are feasible to make and test. Competing physical processes such as chain folding, intramolecular hydrophobic bonding, micelle formation, etc., impose limits on the length of alkyl side chains to be considered. The maximum number of analogs to be made before an optimal one is found is listed in Table I for various search intervals.

Since we have been discussing a *molecular property* which is roughly proportional to the number of CH_2 or CH_3 groups present, the technique applies not only to straight chains at one particular site but generally to multiple substitutions, chain branching, and cyclic substituents. The only requirement is that there be a maximum within the chosen interval.

In summary, this search method will find the most active analog of any series provided that the biological activity exhibits a maximum with respect to some molecular property. The exact form of the relationship is immaterial. In the case of alkyl substitutions, the molecular property involved is most likely to be the partition coefficient. However, the molecular property need not be considered explicitly since properties such as the partition coefficient or molecular refractivity are roughly proportional to the number of CH_2 or CH_3 groups present.

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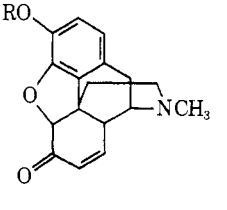
Preparation of Morphine-6-^3H and Its Isotopic Stability in Man and in Rat

Jack Fishman,* Baiba Norton, MaryLou Cotter, and Elliot F. Hahn

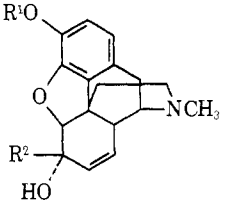
Institute for Steroid Research, Montefiore Hospital and Medical Center, Bronx, New York 10467. *Received February 15, 1974*

Morphine labeled with radioactive isotopes is essential to the detailed study of the biological disposition and biochemistry of this premier narcotic alkaloid. In order to permit tracing and identification of all its transformation products, the isotope in the labeled material must be located in a biologically stable position. To allow for the investigation of specific protein binding of morphine and in particular to aid in the search for the putative narcotic receptor, radioactive substrates with high specific activity are required. In addition, the recent advent of radioimmunoassay for morphine creates the need for high specific activity radiolabeled material.[1,†] Presently available labeled morphines have severe limitations by the above criteria. The initial radiolabeled alkaloid, morphine-*N*-$^{14}CH_3$,[2] suffers loss of the isotope in the course of N-demethylation, a significant biotransformation of morphine.[3] The specific activity of the material is also limited by the nature of the isotope to a maximum of about 50 mCi/mmol. Biological stability limitations apply also to the recently described morphine-*N*-CT_3 which has been prepared with a specific activity of 68 mCi/mmol.[4] Randomly labeled morphine-^3H has been obtained *via* tritium exchange by several procedures including acid catalysis[5] and microwave irradiation.[6] While apparently biologically stable, these substrates have reported maximum specific activities of less than 10 mCi/mmol rendering them of limited value for the purposes detailed above. Selective tritium introduction into the morphine molecule has been achieved in the preparation of morphine-2-^3H,[7,8] but the specific activity of the product is only of the order of 12 mCi/mmol.

Chemistry. It appeared to us that the best route to high specific activity radiolabeled morphine required the specific introduction of tritium by means of a one-step



Ia = H
b = Ac
c = CH_3



IIa, R^1 = Ac; R^2 = H
b, R^1 = Ac; R^2 = ^3H
c, R^1 = H; R^2 = ^3H
d, R^1 = CH_3; R^2 = ^3H

reaction on a suitable substrate. The reduction of the 6-keto derivative of morphine, morphinone (Ia),[9] with a labeled reagent to effect introduction of tritium into the 6β

† In the absence of high specific activity morphine, dihydromorphine-7,8-^3H (sp act. 388 mCi/mmol) was used as a substitute.

position of the morphine molecule provided such a sequence. The location of the isotope, however, made it vulnerable to any metabolic oxidation of 6-hydroxyl group and a demonstration of the biological stability of the tritium in morphine-6-^3H was essential to the success of this effort.

Morphine (Ia) is a highly labile substance and both its generation and subsequent elaboration required a protective group on the phenolic hydroxyl at C-3. For the purposes of our work we selected the acetate ester rather than the methoxymethyl ether previously employed in the synthesis of morphinone.[9] Selective monoacetylation of morphine to yield the 3-monoacetate was reported by Welsh.[10] We have found that an equivalent of acetic anhydride in pyridine at room temperature gave mostly morphine 3-acetate (IIa) with only a minor contamination of the diacetate. The structure of the resin-like product followed from its infrared spectrum which was identical with that of the previously prepared material.[10] In addition, the nmr spectrum of the product was in accord with the morphine 3-acetate (IIa) structure with a single acetate methyl resonance at 2.28 ppm, the 6β proton appearing as a broad multiplet at 4.18 ppm and the C-5 proton as a doublet at 4.86 ppm.

The oxidation of morphine 3-acetate to morphinone acetate (Ib) was accomplished with activated manganese dioxide in chloroform. The morphinone acetate (Ib) was a homogenous material which resisted crystallization. Its infrared spectrum exhibited conjugated carbonyl absorption at 1680 cm^{-1}. In the nmr spectrum the C-5 proton now appeared as a singlet at 4.70 ppm, confirming the loss of the 6-hydrogen and the C-7,8 proton resonance was shifted downfield and was centered at 6.32 ppm. Reduction of the morphinone acetate (Ib) with sodium borohydride in ethanol was accompanied by simultaneous hydrolysis of the 3-acetate to regenerate morphine in good yield.

When the above reduction was repeated with sodium borohydride-^3H with a specific activity of 250 mCi/mmol, morphine-6-^3H (IIc) was obtained which, after chromatographic purification, was 97% radiohomogenous by reverse isotope dilution analysis with carrier morphine. To ensure the specificity of the tritium location morphine-6-^3H diluted with carrier was converted to codeine with diazomethane and oxidized to codeinone (Ic) with manganese dioxide. The oxidation resulted in a loss of 99.6% of the radioactivity, indicating that this percentage of tritium was located at C-6.

Isotopic Stability of Morphine-6-^3H. The oxidation of primary and secondary hydroxyl functions is a common metabolic transformation of most substrates. Such an oxidation of morphine to morphinone has been postulated as one explanation for the persistent binding of the alkaloid to brain proteins.[11] Nevertheless, products of the biological oxidation of the 6-hydroxyl group in morphine have not been detected in either *in vitro* or *in vivo* studies. This failure, in itself, does not exclude the presence of the reaction since the extreme instability of the oxidation products[9] may have prevented their isolation. Furthermore, the oxidation of morphine could be a reversible process thus effectively removing evidence of the reaction. Neither of the above reservations applies to the present studies where the existence and extent of the oxidation is monitored not *via* its products but from a water by-product of the reaction, since by analogy to other similar oxidations[12] it may be assumed that any biological oxidation of the 6-hydroxyl group of morphine-6-^3H would result in the formation of tritiated water. Morphine-6-^3H was injected into rats, blood was obtained at 1 and 6 hr after injection, frozen, and lyophilized, and the water so obtained was counted. As a control a similar quantity of morphine-6-^3H was incubated in phosphate buffer for the same periods of time and the incubation water was analyzed for isotope exchange. The data obtained are listed in Table I and show that there is a small time-dependent nonenzymatic exchange of tritium from morphine-6-^3H. This may derive from an allylic rearrangement process or from the 0.4% of tritium located elsewhere than C-6. Correcting for this effect the total amount of tritium released from morphine-6-^3H into the estimated 150 ml of body water in the rat corresponds at the highest to 0.1% of the dose.

Table I. ^3H_2O Derived from Morphine-6-^3H *in Vivo* as Per Cent of Dose

	1 hr	6 hr	24 hr
^3H_2O in total body water (rat)[a]	0.5	1.1	
^3H_2O in total body water (man)[b]		1.3	1.3
^3H_2O in control incubation[c]	0.4	1.0	

[a] Calculated at 150 ml per 300 g wt. [b] Calculated at 48 l. for 74 kg wt. [c] Morphine-6-^3H 224,000 cpm in 150 ml of Tris, pH 7.2, buffer at 37°.

The possibility that the enzymatic loss of tritium is not significant in whole body metabolism but may be meaningful in specific tissues prompted us to incubate morphine-6-^3H with fresh rat brain and liver tissue along with an appropriate control. The water obtained from the incubations revealed an exchange of 0.07% of the substrate for the control, and 0.15 and 0.13% for the brain and liver incubations, respectively. Therefore, the release of tritium for morphine-6-^3H in the presence of brain and liver homogenates was only 0.08 and 0.06% of the substrates, indicating isotopic stability of the substrate in these *in vitro* conditions.

The biologic stability of the isotope of morphine-6-^3H in man was demonstrated when a tracer dose of morphine-6-^3H was injected intravenously into a volunteer male patient. The tritiated water derived from both blood and urine samples obtained at intervals for 72 hr, when extrapolated to the total body water volume, totaled 1.3% of the dose administered. When corrected for the nonenzymatic tritium exchange this represents an exchange of at most only 0.3% of the administered dose. The probable existence of a primary isotope effect in this type of a reaction[13] would serve to diminish the oxidation of tritium-containing molecules and therefore mask to a certain extent the quantitative significance of the reaction. However, even correcting for such an isotope effect, these results would still prove only minimal values for the oxidation of morphine at C-6 in biological systems and demonstrate adequately the absence of the reaction. The fact that these results were observed both *in vivo* and *in vitro* suggests that rapid excretion and conjugation of morphine at C-6[14] is not the sole explanation for the lack of oxidation of the allylic hydroxyl group of morphine but that its absence is probably the result of a lack of an oxidoreductase enzyme utilizing morphine as a substrate.

To ensure that morphine-6-^3H would be suitable for metabolic studies it was necessary to demonstrate isotopic stability under the fairly drastic conditions frequently employed for morphine conjugate hydrolysis. When morphine-6-^3H and morphine-N-$^{14}CH_3$ were autoclaved in 10% hydrochloric acid the recovery of radioactivity in both instances was essentially identical, demonstrating the absence of any selective isotope loss from morphine-6-^3H. The biological and chemical stability of the isotope in morphine-6-^3H is of considerable significance in that it permits the preparation of high specific activity radioactive morphine for biological experimentation. Sodium bor-

Table II. Cumulative Urinary Radioactivity (% Dose) in Men after iv and im Administration of Morphine-6-^3H

Urine collection, hr	Subject 1, iv	Subject 2, im
6	48.4	41.2
12	64.2	60.1
24	75.3	75.2
48	81.5	83.8
72	83.2	87.2

ohydride with a specific activity of 80 Ci/mmol is available and allows the synthesis of morphine-6-^3H with a specific activity of up to 20 Ci/mmol. This is orders of magnitude greater than any heretofore available and provides material suitable for high sensitivity radioimmunoassays and, more importantly, for the investigation of the subcellular disposition and biochemistry of this narcotic alkaloid. The stability of the isotope in morphine-6-^3H under the vigorous acid autoclave conditions also gives assurance that there will be minimal loss of the isotope during any of the isolation and separation steps involved in the work-up of biological fluids and tissues.

We have used the above morphine-6-^3H to compare the rate and extent of urinary excretion in man after either intravenous or intramuscular administration of the alkaloid. The results of these studies are recorded in Table II and show that irrespective of the injection route the excretion in urine was rapid and extensive. In both cases about 60% of the dose was excreted within the first 12 hr and the total at the end of 72 hr corresponded to about 85% of the dose. The latter value is significantly higher than the average results previously obtained in studies in which morphine-*N*-methyl-^{14}C was used. In these studies the amount of ^{14}C expired as CO_2 was also measured but it is insufficient to compensate for the smaller urinary excretion, suggesting that not all of the isotope lost *via* N-demethylation can be accounted for in expired CO_2.

Experimental Section‡

Morphine 3-Acetate (IIa). To a solution of 200 mg (0.70 mmol) of morphine in 1.5 ml of pyridine, 0.075 ml (0.73 mmol) of acetic anhydride was added. The mixture was allowed to stand overnight at room temperature after which time it was quenched with ice and diluted with 25 ml of water. The reaction was then extracted four times with 50 ml of chloroform, and the combined organic extract was backwashed four times with 25 ml of water. After drying over anhydrous sodium sulfate the solvent was removed under reduced pressure. The residue was a clear resin-like material which could not be crystallized. Thin-layer chromatography of an aliquot of this product on silica gel in the system methanol–chloroform (25:75) containing 2 drops of NH_4OH revealed one principal spot, which ran midway between standards of heroin and morphine, and a very faint spot, estimated at less than 2%, which was coincident with heroin. The infrared spectrum of the product was identical with that of morphine 3-acetate.[10] Its nmr spectrum revealed resonances for the acetate CH_3 and NCH_3 as singlets at 2.28 and 2.40 ppm, respectively. The C-5 proton appeared as a doublet centered at 4.86 ppm while the 6β H resonated as a broad multiplet at 4.18 ppm. In contrast, the 6-acetate methyl of heroin absorbs at 2.18 ppm.

Morphinone Acetate (Ib). A solution of 116 mg of morphine 3-acetate (IIa) in 10 ml of chloroform was stirred for 3 hr at room temperature with 515 mg of activated manganese dioxide.§ The reagent was filtered off and washed repeatedly with absolute ethanol. The combined filtrate and washings were evaporated and the residue was purified by preparative thin-layer chromatography on silica in the system methanol–ethyl acetate (25:75). The main ultraviolet absorbing zone somewhat less polar than morphine 3-acetate was eluted with chloroform–2-propanol (3:1) to yield 74 mg of morphinone acetate (Ib) as a homogenous oil which resisted crystallization. The nmr spectrum showed resonances for the C-5 proton as a singlet at 4.70 ppm and the C-8 portion of the C-7,8 multiplet appeared at 6.02 ppm as a quartet, $J_{7,8}$ = 5, $J_{8,14}$ = 1 Hz. The C-7 resonance was hidden under the aromatic proton absorption at 6.8 ppm. The 6β H resonance was missing and the *N*-methyl and acetate methyl absorptions were at 2.40 and 2.24 ppm, respectively. The infrared spectrum exhibited a carbonyl absorption at 1680 cm^{-1}.

A solution of 20 mg of morphinone acetate (Ib) in 3 ml of ethanol was stirred with 20 mg of sodium borohydride for 2 hr. The usual work-up yielded 12 mg of morphine identical in all respects with the authentic material.

Morphine-6-^3H (IIc). A solution of 28 mg of morphinone acetate (Ib) in 3 ml of absolute ethanol was stirred at room temperature with 5 mg of sodium borohydride-^3H (specific activity 250 mCi/mmol). At the end of the 2 hr 3 ml of ice-cold 5% hydrochloric acid was added dropwise. The pH was then adjusted to 9 with dilute ammonium hydroxide and the mixture was extracted four times with 15 ml of chloroform. The chloroform extract was dried and evaporated, and the labile tritium was removed from the residue by repeatedly dissolving in ethanol and removing the solvent *in vacuo*.

The crude radioactive product was purified by preparative thin-layer chromatography in the system chloroform–methanol (6:4) containing a few drops of NH_4OH. The plate was visualized under long-wave ultraviolet and absorbing zones corresponding to morphine and morphine 3-acetate were scraped off. The morphine region (R_f 0.23) was eluted with ethanol. The purified radioactive material was also coincident with morphine in the system chloroform–methanol (9:1). No evidence for the presence of any 6-iso-morphine was found.

An aliquot of the purified morphine-6-^3H containing 325,000 cpm was diluted with 42.3 mg of carrier morphine. Successive crystallizations from methanol–acetone gave crystals with specific activities of 7430, 7640, and 7550 cpm/mg indicating that 97% of the radioactivity was associated with morphine. The specific activity of the purified undiluted morphine-6-^3H was 58 mCi/mmol which corresponds well with the specific activity of the reagent and the stoichiometry of the borohydride reduction.

Proof of Isotope Location in Morphine-6-^3H. To confirm the location of the isotope in morphine-6-^3H, a 400-mg sample of the material, specific activity 4.54 × 10^5 cpm/mmol, was dissolved in 100 ml of 95% ethanol and 100 ml of methanol and allowed to stand with excess ethereal diazomethane overnight. The solvents were removed; the residue was taken up in 50 ml of chloroform and washed well with 2 *N* NaOH. After drying the organic layer was evaporated and codeine-6-^3H was crystallized from ethyl acetate. The specific activity of the codeine was 4.63 × 10^5 cpm/mmol, serving as a further check of the radiohomogeneity of the morphine-6-^3H. Codeine-6-^3H (300 mg) was dissolved in 25 ml of methylene chloride and stirred at room temperature for 1 hr with 1.2 g of activated manganese dioxide. Filtration and evaporation of the solvent gave a residue which was crystallized from ethyl acetate to give 148 mg of codeinone, mp 180–182° (lit.[16] mp 181.5–182.5°). The specific activity of the material upon successive crystallizations was 5, 6, 4 cpm/mg or 0.015 × 10^5 cpm/mmol. This corresponds to a loss of 99.6% of the original radioactivity and therefore this percentage of the isotope in morphine-^3H was located at C-6.

Biologic Isotope Stability of Morphine-6-^3H *in Vivo* and *in Vitro* in the Rat. Morphine-6-^3H (1 mg, 224,000 cpm) was administered intramuscularly as the hydrochloride to two male rats weighing 300 g each. The rats were sacrificed at 1 and 6 hr after injection and their blood was collected, frozen, and lyophilized. The water so obtained was counted as three 1-ml aliquots in Diotol. As a control an identical quantity of morphine-6-^3H was incubated in 150 ml of Tris buffer, pH 7.2, at 37°, 10-ml samples were removed at 1 and 6 hr, frozen, and lyophilized, and the specific activity of the water was determined. Data obtained are listed in Table I.

Fresh brain and liver tissue obtained from male rats was homogenized in Tris buffer, pH 7.2, in an all-glass homogenizer. Aliquots of the homogenates corresponding to 1 g of tissue were incubated at 37° with morphine-6-^3H (50 μg, 220,000 cpm) in the presence of NAD (25 μM), glucose 6-phosphate (80 μM), and glu-

‡ Melting points are uncorrected. The infrared spectra were obtained in KBr. The nmr spectra were obtained in deuteriochloroform on a Varian spectrometer. Where analyses are indicated by the symbols of the elements, the analytical results for these elements were within 0.3% of the theoretical values.

§ This reagent was found to be preferable to the silver carbonate used by Rapoport, *et al.*, ref 9.

cose-6-phosphate dehydrogenase (20 units). As a control an identical incubation was performed with liver homogenate which had been heated previously for 15 min in boiling water. The incubations were terminated after 30 min by the addition of 3 ml of water and freezing. Water obtained from each incubation by lyophilization was counted in Diotol as three 1-ml aliquots. The total tritium water content of the brain and liver incubations was 0.15 and 0.13% of the substrate, while the control incubation contained 0.07%. Therefore, the release of tritium from morphine-6-^3H in the presence of brain and liver homogenates was only 0.08 and 0.06% of the material incubated.

Isotopic Stability of Morphine-6-^3H in Man. A volunteer 27-year-old male subject weighing 74 kg was given an intravenous injection of morphine-6-^3H (100 μg, 7.2×10^6 cpm). A blood sample was obtained at 6 hr after injection and urine was collected for 72 hr at intervals. The blood and aliquots of the urine collections were frozen and lyophilized and the water thus obtained was counted. Both plasma and urinary water had a maximum specific activity of 2 cpm/ml. Since the estimated total body water of a 74-kg man is 48 l., the total body water tritium content is 96,000 cpm or 1.3% of the dose. When corrected for the control, the total exchange amounts to 0.3% of the injected material.

Isotope Stability of Morphine-6-^3H under Acid Hydrolysis Conditions. Morphine-6-^3H (0.25 mg, 612,000 cpm) was dissolved in 10 ml of 10% hydrochloric acid and heated at 121–124° at 18 atm of pressure for 60 min.[17] The solution was cooled, the pH adjusted to 9.0 with base, and the basic solution extracted four times with chloroform–2-propanol (3:1). An identical experiment was performed with morphine-N-$^{14}CH_3$ (0.25 mg, 153,000 cpm).

The recovery of radioactivity in the organic extract was 39.8% for morphine-6-^3H and 38.7% for morphine-N-$^{14}CH_3$. The similar recoveries demonstrate that the acid conditions did not lead to selective isotope loss from morphine-6-^3H.

Urinary Excretion of Morphine-6-^3H in Men after iv and im Injection. Morphine-6-^3H (7.16×10^6 cpm, 0.1 mg) in redistilled propylene glycol was administered intravenously to a 27-year-old volunteer male subject. Urines were collected at intervals for 72 hr. Aliquots of each urine collection were diluted with water and counted in Diotol on a Packard liquid scintillation counter with suitable quenching corrections. Other aliquots of each urine collection were lyophilized to obtain water which was counted.

In the second study morphine-6-^3H (7.4×10^6 cpm, 10 mg) in propylene glycol was administered intramuscularly to a 24-year-old male volunteer subject. The urine collection and counting were then carried out precisely as in the previous study.

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Synthesis and Some Pharmacological Properties of 8-L-Homoarginine-vasopressin and of 1-Deamino-8-L-homoarginine-vasopressin

Gunnar Lindeberg, Miklos Bodanszky,* Margot Acosta, and Wilbur H. Sawyer

Biokemiska Institutionen, University of Uppsala, Uppsala, Sweden, Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106, and Department of Pharmacology, Columbia University, College of Physicians and Surgeons, New York, New York 10032. *Received January 11, 1974*

Syntheses of 8-L-homolysine-vasopressin[1] and its 1-deamino derivative[2] were reported from these laboratories. The high biological activity† of the homolysine analogs in the rat prompted the preparation of the corresponding cyclic peptides with L-homoarginine in position 8. It seemed to be reasonable to expect that again potent compounds can be obtained. Indeed, both homoarginine analogs are highly active by several biological assays (Table I). 8-L-Homoarginine-vasopressin has somewhat greater antidiuretic activity[4] but slightly lower vasopressor activity[5] than does 8-L-arginine-vasopressin. 1-Deamino-8-L-homoarginine-vasopressin also has greater antidiuretic activity than 8-L-arginine-vasopressin but considerably less than 1-deamino-8-L-arginine-vasopressin. Its vasopressor activity is also lower than that of the L-arginine analogs. The oxytocic (isolated rat uterus) activities[6] of the L-homoarginine analogs are quite similar to those of the corresponding L-arginine analogs.

The pharmacological properties of the new vasopressin analogs demonstrate that the precise length of the side chain in position 8 is not critical to antidiuretic and vasopressor activities, as shown by the ornithine-lysine-homolysine series[2] and the arginine-homoarginine pair. The basic character of this side chain is more important.

The synthesis of the two new hormone analogs was carried out in solution according to the stepwise strategy.[7,8] The guanidine group in the side chain of the homoarginine residue was protected by tosylation.[9,10] For the coupling steps, the dicyclohexylcarbodiimide method[11] and *p*-nitrophenyl esters of the protected amino acids[7] were used. After completion of the chain elongation, the protecting groups were removed by reduction with sodium in liquid ammonia and the cyclic disulfides were formed by oxidation with air[12] or potassium ferricyanide. The new analogs were obtained in homogeneous form by a combination of gel filtration, ion-exchange chromatography, and partition chromatography.

*Author to whom correspondence should be addressed at Case Western Reserve University.

†A reexamination of the activities of 1-deamino-8-L-homolysine-vasopressin confirmed that it is a potent analog of the parent hormone but also showed that the previously reported (ref 2) values require considerable corrections (*cf.* Table I and ref 3).