studies of Cox and Osman [9], chloramphenicol had no effect on the development of tolerance to morphine.

The studies here indicate that morphine can cause a very rapid development of tolerance to calcium depletion. The blockade of this effect by a protein synthesis inhibitor, cycloheximide, indicates that morphine may be inducing a rapid alteration of membrane protein synthesis in producing tolerance to calcium depletion.

DAVID H. ROSS
SHERWOOD C. LYNN*

Departments of Pharmacology and Psychiatry, University of Texas Health Science Center, San Antonio, Texas 78284, U.S.A.

* Recipient of an NIMH Psychiatry Training Grant Medical Student Fellowship.

REFERENCES

1. D. H. Ross, M. A. Medina and H. L. Cardenas, *Science, N.Y.* **186**, 63 (1974).
2. J. Glowinski and L. L. Iverson, *J. Neurochem.* **13**, 655 (1966).
3. A. A. Smith, in *Narcotic Drugs: Biochemical Pharmacology* (Ed. D. H. Clouet), p. 424.
4. E. L. Way and F. Shen, in *Narcotic Drugs: Biochemical Pharmacology* (Ed. D. H. Clouet), p. 229. Plenum Press, New York (1971).
5. R. A. Verri, F. G. Graeff and A. P. Carrodo, *Int. J. Neuropharmac.* **7**, 293 (1968).
6. F. H. Shen, H. Loh and E. L. Way, *Fedn Proc.* **28**, 793 (1969).
7. A. Smith, K. Lisper and K. Hayashida, *Fedn Proc.* **28**, 261 (1969).
8. H. H. Loh, Fu-A. Shen and E. L. Way, *Biochem. Pharmac.* **18**, 2711 (1969).
9. B. M. Cox and B. H. Osman, *Br. J. Pharmac. Chemother.* **38**, 157 (1970).
10. I. G. Morgan and L. Austin, *J. Neurochem.* **15**, 41 (1968).

Biochemical Pharmacology, Vol. 24, pp. 1137–1139. Pergamon Press, 1975. Printed in Great Britain.

Formation of angiotensin II from tetradecapeptide renin substrate by angiotensin-converting enzyme*

(*Received* 21 *September* 1974; *accepted* 29 *October* 1974)

Tetradecapeptide renin substrate (TDP, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser) was first isolated from a partial tryptic digest of renin substrate (angiotensinogen) [1]. Although TDP can serve as a substrate for both renin [2] and pseudorenin [3], it has not been shown to occur naturally. In the anesthetized, pentolinium-treated rat in our laboratory, TDP elicits a pressor response which is different from that of angiotensin I both in intensity (1:50†) and duration (2×). The ratio of pressor activity of TDP relative to angiotensin II has been reported to be 1:37 [4], while the ratio of contractile activity in the isolated rat colon has been reported as 1:9 [5]. A ratio of 1:15 has been reported for the stimulation of release of catecholamines from the isolated cat adrenal gland [6]. Montague *et al.* [7] have reported a TDP:angiotensin II ratio of 1:12 for both pressor activity in the rat and contractile activity of isolated guinea pig ileum. It is not known whether these biological activities are due to the complete 14-residue sequence of TDP or are a function of a smaller peptide (e.g. angiotensin II) produced by hydrolysis of TDP.

Angiotensin-converting enzyme has been shown to act on a wide variety of peptide substrates *in vitro* by removing dipeptide units from the C-terminus of the peptide chain [8]. Our present study reports the finding that angiotensin-converting enzyme can form angiotensin II from TDP by the successive removal of three dipeptides: Tyr-Ser, Leu-Val and His-Leu. Since the enzyme cannot hydrolyze a peptide bond involving the imino group of proline [9], further hydrolysis is prevented, and angiotensin II is the limit peptide.

TDP and the nonapeptide (His^6-Ser^{14})‡ were synthesized by the solid phase method as described previously [3], and angiotensin-converting enzyme was purified from hog lung [10]. His-Leu was synthesized in this laboratory [11], Leu-Val was purchased from Fox Chemical Co. and Tyr-Ser was made by reducing Cbz-Tyr-Ser (Cyclo Chemical Co.) with H_2/Pd. The concentrations of solutions of the peptides were determined by amino acid analysis after acid hydrolysis. The synthetic peptide < Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro was a gift from Dr. J. W. Ryan, Papanicolaou Cancer Research Institute. Biological assays were performed in the anesthetized, pentolinium-treated rat, using angiotensin I as a standard as previously described [12].

Initial reaction rates were determined with a Technicon Auto Analyzer using the ninhydrin-reaction procedure described previously [13]. This procedure measures continuously the formation of new free amino groups as peptide bonds are hydrolyzed. TDP ($9{\cdot}2 \times 10^{-6}$ M) was incubated at 37° with converting enzyme in 0·05 M sodium-Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7·5, with NaCl at the indicated concentration in a total volume of 10 ml. The incubation mixtures were sampled into the AutoAnalyzer continuously for 18 min, and enzyme velocities were calculated from the slopes of the recordings. Rates are expressed as μmoles dipeptide formed/min/mg of enzyme in terms of leucine color equivalents. Color values of the dipeptides relative to leucine (100) are: Tyr-Ser, 87; Leu-Val, 90; and His-Leu, 52. Hydrolysis rates for the nonapeptide (His^6-Ser^{14}) were determined in a similar manner.

The initial velocity of TDP hydrolysis by converting enzyme is dependent on chloride, as shown in Table 1.

* This research was supported by the Veterans Administration (Project No. 7963–01).

† Ratios of biological activity of TDP:angiotensin have been calculated on a molar basis.

‡ Peptide fragments of TDP are numbered according to their position in the TDP sequence.

Table 1. Effect of NaCl on the hydrolysis of peptides by angiotensin-converting enzyme.

NaCl concn (M)	Initial velocity* (μmoles/min/mg) TDP	Nonapeptide (His^6-Ser^{14})	Angiotensin I†
0·0	0·02	0·34	0·03
0·001	0·04	1·4	
0·01	0·09	1·7	0·50
0·1	0·13	1·4	1·2

* Substrate concentration was 9.2×10^{-6} M for TDP, 11×10^{-6} M for nonapeptide, and 10×10^{-6} M for angiotensin I; other experimental details are given in the text.

† Data for angiotensin I are taken from Ref. 14.

The pattern of chloride dependence for TDP is similar to that for angiotensin I; both show very little hydrolysis in the absence of added NaCl. At all chloride concentrations, the rate of hydrolysis of TDP is less than that of angiotensin I. The nonapeptide (His^6-Ser^{14}) is hydrolyzed much faster than TDP and shows a pattern of chloride dependence similar to that found for bradykinin [14]. In fact, there is a striking similarity if one compares TDP with the nonapeptide (His^6-Ser^{14}) on the one hand, and Gly-Arg-Met-Lys-bradykinin (a tridecapeptide) with bradykinin (a nonapeptide) on the other hand [14]. In both cases, the elongation of the peptide chain at the N-terminus produces the same change in the pattern of chloride dependence: a marked decrease in the rate of hydrolysis at all chloride concentrations and a shift in maximal activity from 0·01 to 0·1 M.

In the presence of 0·1 M NaCl, the hydrolysis of TDP is completely inhibited by 5×10^{-6} M < Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro, a peptide found in *Bothrops jararaca* venom which is known to inhibit conversion of angiotensin I to II [8].

The conversion of TDP by converting enzyme to a more potent pressor substance is illustrated in Fig. 1. The negligible increase in pressor activity in the absence of NaCl and the pattern of activation by NaCl parallel the above results obtained with the AutoAnalyzer. Figure 2 shows the time-course of the hydrolysis of TDP by converting enzyme. As noted above, hydrolysis in the absence of chloride is very slow. The final value, indicating that three bonds are being hydrolyzed, is in agreement with the previous finding that the enzyme does not attack angiotensin II [8]. In order to identify the products of the reaction, 184 nmoles TDP was incubated for 2 hr at 37° with 20 μg converting enzyme at pH 7·5 in the presence of 0·05 M NaCl in a total volume of 2 ml. The reaction was stopped by boiling and acidifying to pH 2·5. Chromatography of the incubation mixture on the amino acid analyzer showed three peaks, corresponding to Tyr-Ser (184 nmoles), Leu-Val (128 nmoles) and His-Leu (99 nmoles). No free amino acids were found. The relative amounts of the three dipeptides found are in agreement with the expected order of release of dipeptide units starting from the C-terminal end of TDP.

Fig. 1. Conversion of TDP to a more potent pressor substance by angiotensin-converting enzyme. TDP (9.2×10^{-6} M) was incubated under the indicated conditions for 30 min at 37° as described in the text. Aliquots were diluted 50-fold, and assayed for pressor activity by injecting 0·25 ml (corresponding to 46 pmoles TDP) into an anesthetized rat [12]. Angiotensin I standards are shown for comparison. (1) 6 pmoles angiotensin I standard; (2) TDP, no enzyme, no NaCl; (3) TDP, 20 μg enzyme, no NaCl; (4) TDP, no enzyme, 0·01 M NaCl; (5) TDP, 20 μg enzyme, 0·01 M NaCl; (6) TDP, no enzyme, 0·1 M NaCl; (7) TDP, 20 μg enzyme, 0·1 M NaCl; (8) 6 pmoles angiotensin I standard; and (9) 2 pmoles angiotensin I standard.

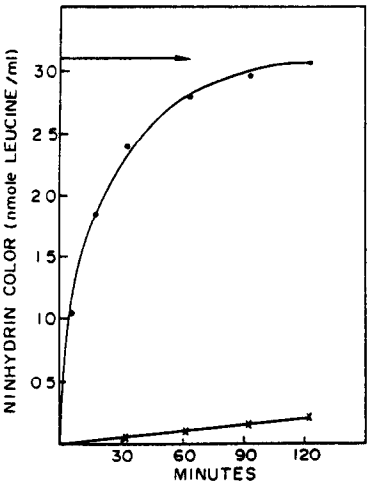

Fig. 2. Hydrolysis of TDP by angiotensin-converting enzyme. TDP (1.35×10^{-6} M) was incubated with 120 μg converting enzyme, both in the presence of 0·1 M NaCl (●——●) and in the absence of NaCl (× —— ×). Incubation conditions were as described in the text except that sampling into the AutoAnalyzer was done discontinuously, at the indicated times. The arrow indicates the total ninhydrin color expected when all three dipeptides (Tyr-Ser, Leu-Val and His-Leu) have been released.

The ability of angiotensin-converting enzyme to hydrolyze TDP in the manner shown above conforms with the previously described dipeptidyl carboxypeptidase-type of specificity of this enzyme [8].

Attempts have been made by other investigators to utilize TDP as a renin substrate for the specific detection or assay of renin activity in tissue extracts or in organ perfusion studies [15–18]. It has previously been shown that TDP is readily hydrolyzed by pseudorenin to form angiotensin I [3]. The present finding that converting enzyme can form angiotensin I and II from TDP should serve as an additional warning against the use of TDP for the detection or assay of renin activity.

Department of Medicine and Surgery, Veterans Administration Hospital, Cleveland, Ohio, and the Departments of Biochemistry and Pathology, Case Western Reserve University, Cleveland, Ohio 44106, U.S.A.

FREDERIC E. DORER
JOSEPH R. KAHN
KENNETH E. LENTZ
MELVIN LEVINE
LEONARD T. SKEGGS

REFERENCES

1. L. T. Skeggs, Jr., J. R. Kahn, K. E. Lentz and N. P. Shumway, *J. exp. Med.* **106**, 439 (1957).
2. L. T. Skeggs, K. E. Lentz, J. R. Kahn and H. Hochstrasser, *J. exp. Med.* **128**, 13 (1968).
3. L. T. Skeggs, K. E. Lentz, J. R. Kahn, F. E. Dorer and M. Levine, *Circulat. Res.* **25**, 451 (1969).
4. W. K. Park, C. Choi, F. Rioux and D. Regoli, *Can. J. Biochem.* **52**, 106 (1974).

5. R. Law, *Nature New Biol.* **231**, 186 (1971).
6. M. J. Peach, F. M. Bumpus and P. A. Khairallah, *J. Pharmac. exp. Ther.* **176**, 366 (1971).
7. D. Montague, B. Riniker, H. Brunner and F. Gross, *Am. J. Physiol.* **210**, 591 (1966).
8. Y. S. Bakhle, in *Angiotensin* (Eds. I. H. Page and F. M. Bumpus), p. 41. Springer, Berlin (1974).
9. Y. E. Elisseeva, V. N. Orekhovich, L. V. Pavlikhina and L. P. Alexeenko, *Clinica. chim. Acta* **31**, 413 (1971).
10. F. E. Dorer, J. R. Kahn, K. E. Lentz, M. Levine and L. T. Skeggs, *Circulat. Res.* **31**, 356 (1972).
11. F. E. Dorer, L. T. Skeggs, J. R. Kahn, K. E. Lentz and M. Levine, *Analyt. Biochem.* **33**, 102 (1970).
12. L. T. Skeggs, Jr., J. R. Kahn and W. H. Marsh, *Lab. Invest.* **2**, 109 (1953).
13. F. E. Dorer, J. R. Kahn, K. E. Lentz, M. Levine and L. T. Skeggs, *Circulat. Res.* **34**, 824 (1974).
14. F. E. Dorer, J. W. Ryan and J. M. Stewart, *Biochem. J.* **141**, 915 (1974).
15. F. A. Mendelsohn and C. I. Johnston, *Biochem. J.* **121**, 241 (1971).
16. D. Ganten, A. Marquez-Julio, P. Granger, K. Hayduk, K. P. Karsunky, R. Boucher and J. Genest, *Am. J. Physiol.* **221**, 1733 (1971).
17. A. B. Gould, G. Keighley and P. H. Lowy, *Lab. Invest.* **18**, 2 (1968).
18. H. D. Itskovitz, L. A. Hebert and J. C. McGiff, *Circulat. Res.* **32**, 550 (1973).

Biochemical Pharmacology, Vol. 24, pp. 1139–1142. Pergamon Press, 1975. Printed in Great Britain.

Inhibition of synaptosomal uptake of choline by various choline analogs

(*Received* 15 *August* 1974; *accepted* 16 *October* 1974)

Choline appears to be transported into cholinergic neurons by a high affinity mechanism [1–3] that is selectively localized to cholinergic nerve terminals [4]. Recent results indicate that this uptake mechanism plays a rate-limiting role in sustaining and regulating the synthesis of acetylcholine [5–7]. Thus, the ability to utilize and manipulate this uptake mechanism provides investigators with a very powerful tool for studying various aspects of acetylcholine synthesis, storage and release. To further a fundamental understanding of this uptake mechanism, we have examined the inhibition of synaptosomal choline uptake by choline analogs and some other compounds known to affect cholinergic systems.

dl-α-Methylcholine, *d*-β-methylcholine, 3-trimethylamino-propan-1-ol (homocholine) and *N*-hydroxyethyl pyrrolidinium (pyrrolidine choline) were prepared as iodide salts by reacting the corresponding tertiary amino alcohols with methyl iodide.

The reactions took place at room temperature overnight in the dark. The compounds were purified by recrystallization from acetone–methanol–ether mixtures. Homogeneity was ascertained by chromatography (*n*-butanol–methanol–acetic acid–water, 8:2:1:3) or electrophoresis at 1000 volts for 30 min (1·5 M acetic acid–0·75 M formic acid) on cellulose thin-layer plates (Chromagrams, Eastman Chemicals, Rochester, N.Y., lot Nos. 5102 and 5133).

We are grateful to Dr. C. Y. Chiou for his generous gift of diethylcholine, and to Dr. Darwin Cheney for his generous gift of 2-hydroxy-1,1,2,2-d_4-ethyl-trimethylammonium (D_4 choline) and 2-hydroxyethyl, d_9-trimethyl ammonium (D_9 choline). Triethylcholine, diethylethanolamine, *N*-methylethanolamine, hemicholinium-3, hemicholinium-15 and oxotremorine were purchased from Aldrich. Choline, 2-aminoethanol, carbamylcholine and phosphorylcholine were from Sigma, and *N*-hydroxyethyl-4-(1-naphthylvinyl) pyridinium (NVP), *d*-tubocurarine and physostigmine were purchased from Calbiochem. Acetylcholine was obtained from Calbiochem, Sigma and Aldrich, and thiocholine was supplied by Polysciences and K & K Labs. Tetramethylammonium and tetraethylammonium were from Baker and *t*-butylethanolamine was from K & K Labs. Other drugs were obtained as follows: BW-284C51 from Wellcome Research Labs; chlorpromazine from Smith Kline & French; haloperidol from McNeil; and neostigmine from Hoffman La-Roche. At least two IC_{50} determinations were made with each compound from each source.

Choline uptake was performed as previously described [4] with some modifications. These procedures are stated briefly as follows. Sprague–Dawley male rats (180–220 g) were killed by decapitation and the brains were quickly removed to a dish of chilled saline. The striatum was

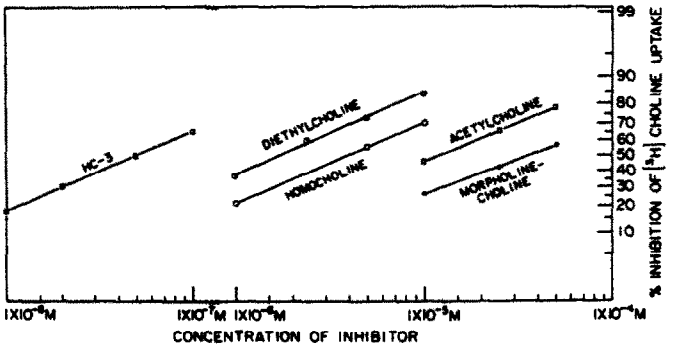

Fig. 1. Log-probit plot of some representative inhibitors of [^{3}H]choline uptake. Crude synaptosomal fractions were incubated for 4 min in the presence of 1 μM [^{3}H]choline and various concentrations of inhibitor. Results are the mean for at least two experiments which differed by less than 10 per cent and are expressed as per cent inhibition of uptake on a probability scale.