Esterase Activity of Chymotrypsin on O-DNP-L-Tyrosine Ethyl Ester Substrates

WHILE developing a chemical method for the assay of ACTH by dinitrophenylation and subsequent hydrolysis with chymotrypsin, we observed that no di-DNP-seryl-tyrosine could be identified in the digestion mixture by gel filtration on 'Sephadex G-25' column and thin layer chromatography. This suggested that DNP-ACTH is attacked by the enzyme, not at the tyrosyl-2 peptide bond, but at other positions yielding large peptide fragments. The O-DNP derivative of tyrosine may interfere with the action of chymotrypsin on the tyrosyl peptide bond. No work seems to have been reported on the esterase activity of chymotrypsin when the phenolic group of tyrosine is blocked by alkylation or acylation. To verify our hypothesis, model substrates were synthesized in which the phenolic group of tyrosine was blocked by a DNP residue.

N-Acetyl-O-DNP-L-tyrosine ethyl ester was synthesized from N-acetyl-L-tyrosine ethyl ester by Sanger's reagent. A colourless crystalline solid was obtained after recrystallization from aqueous ethanol and had the following characteristics: m.p. 113°–114° C; infrared absorbance peaks, in cm^{-1} (KBr): 1,730, 1,740 (carbonyl); 1,650, 1,600 (amide); 1,560, 1,350, 850 (nitro); found C, 54·46; H, 4·44; N, 9·88—$C_{19}H_{19}O_8N_3$ requires C, 54·67; H, 4·58; N, 10·06 per cent. No hydrolysis of this substrate with chymotrypsin could be detected by a potentiometric method, even after incubation for 12 h, while the corresponding free phenolic compound is the usual substrate for chymotrypsin with a K_m value of $6·9 \times 10^{-4}$ M (ref. 1).

Because this substrate was sparingly soluble in aqueous medium, further evidence was sought by synthesizing a soluble substrate, N-maleyl-O-DNP-L-tyrosine ethyl ester. N-maleyl-L-tyrosine ethyl ester was synthesized by maleylation of L-tyrosine ethyl ester using the procedure of Sheehan *et al.*[2]. An oily product was obtained which could not be crystallized, but which was purified by high voltage electrophoresis in a buffer of pyridine–acetic acid–water (25:1:225, *p*H 6·5), at a voltage of 2 kV for 1·5 h. The pure maleyl derivative was eluted from the paper with acetone yielding an oily material. Its purity was checked by high voltage electrophoresis and thin layer chromatography. This material was directly dinitrophenylated in the usual way giving a colourless solid: m.p. 58°–60° (decomp.); infrared absorbance, in cm^{-1}, (KBr) 1,720, 1,740 (carbonyl); 1,650, 1,600 (amide); 1,560, 1,345, 850 (nitro); found C, 53·03; H, 4·40; N, 8·43—$C_{21}H_{19}O_{10}N_3$ requires C, 53·28; H, 4·02; N, 8·87 per cent. N-Maleyl-L-tyrosine ethyl ester was easily cleaved by chymotrypsin (K_m, $2·08 \times 10^{-2}$M), but the corresponding O-DNP derivative resisted hydrolysis.

These experiments strongly suggest that, for chymotryptic cleavage at the tyrosyl bond, the phenolic group must be free. It would be interesting to test model substrates in which the DNP group was replaced by groups having a negative inductive effect with varying degrees of hindrance.

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Marijuana and Immediate Memory

CLINICAL anecdotes and recent studies suggest that a marijuana "high" interferes with immediate memory (ref. 1, and unpublished manuscripts by L. Clark and A. Wikler). We describe here the effects of calibrated amounts of tetrahydrocannabinol (THC) on cognitive tasks which require immediate memory.

We used THC that had been extracted from marijuana supplied by the Bureau of Narcotics and Dangerous Drugs. Thin layer and gas liquid chromatography of these extracts showed predominantly 1-Δ^1-THC, with some cannabidiol and cannabinol. Quantitative calibrations were made so that doses (20, 40 and 60 mg) were based on the actual content of 1-Δ^1-THC. The doses were in the range which, for synthetic 1-Δ^1-THC administered orally, invariably produces clinical effects approaching those of psychotomimetic drugs, such as lysergide[2,3]. A placebo was composed from an extract of marijuana with cannabinoids removed.

The subjects, who were fasting, received their doses in a randomized order unknown either to them or to the testers. Four oral doses, in 95 per cent ethanol diluted with water, were given on four separate experimental days at least one week apart. The eight subjects were college-educated males in their twenties, weighing between 63 and 85 kg, who had been screened for good physical and mental health. All had smoked marijuana, but at no greater frequency than once a month. They were tested before the drug was given, and at 1·5, 3·5 and 5·5 h afterwards. All testing was done individually in an austere research laboratory.

The digit span task required subjects to repeat, either forwards or backwards, a series of random digits read by the experimenter at a rate of one a second. The span was the longest number of digits that could be reproduced without error on two successive test trials. If the subject's attention waned, the investigator restarted the trial to determine accurately the upper limit of the subject's capacity for that test period.

Table 1. DIGIT SPAN AND THC DOSES TESTED BY ANALYSIS OF VARIANCE (d.f. = 3,21).

THC dose	Means: Digits forward	Means: Digits backward
Placebo	9·0	7·8
20 mg	8·2*	6·9*
40 mg	8·1*	6·6*
60 mg	7·9*	6·4*
F	4·5	5·2
Significant	$P < 0·05$	$P < 0·01$

* These values significantly differ from placebo at $P < 0·01$ by *t*-test for correlative means, 2-tail.

Table 1 shows that significant differences between placebo and each of the THC doses were obtained for both forward and backward digit spans. There were, however, no significant differences between effects of 20, 40 and 60 mg doses, indicating that the smaller dose of THC impairs immediate memory and that larger doses do not significantly augment the impairment. The most significant decrement in forward memory occurred 1·5 h after drug ingestion, with a return almost to the pre-administration result at 3·5 h. For backward memory, there was a greater mean decrement over doses and the impairment was still present 3·5 h after ingestion.

We also gave three subjects a more complicated digit reordering test in two stages. We first read a list of random digits, shorter by two numbers than the subject's forward memory span before he had taken the drug that day, and asked him to recite the digits in the same order. We then instructed the subject to arrange the digits in serial rank order, either upwards or downwards. Each of the three subjects was given at least two reordering tasks during the four testing periods of each experimental day. In the non-drug state (with placebo and before taking the drug), subjects made nineteen errors in