away from this area, since the outfall is equipped with diffusers designed to spread and dilute the effluent and since DDT is associated with the finer, more mobile particles which are easily carried away by the strong tidal currents in the area (*6, 13*). This spread of DDT residues from the outfall is indicated by the general decrease of total DDT concentration in *Emerita* from the outfall area to point 3, 573 km away.

Particles in bottom sediments laden with DDT can be resuspended in the water column during storms or periods of upwelling and mixing. Such a resuspension is implied by the increase of total DDT in *Emerita* from November 1970 to February 1971 at points 13, 14, 15, and 16, despite a reported reduction of input into the sewer system (Table 1). The increased DDE/DDT ratio during this period indicates that the animals have taken relatively older residues such as those that have resided in bottom sediments and have been lifted by rough winter seas. Thus this reservoir of well over 100 metric tons is of potential biological importance and should be considered, along with Cox's estimate of 46 metric tons suspended in the photic zone of the California coastal waters, in estimating the total local burden of DDT (*14*).

The maximum DDT value listed in Table 1 does not correspond geographically to the maximum DDE value. Two explanations are plausible. (i) There is a major DDT input, distinct from the DDE input, which is closer to point 14 than to point 15—the Los Angeles City sewer system, which is totally separate from the Los Angeles County system, has an outfall almost directly offshore from point 14. There is no known major input of DDT into the Los Angeles City sewer system; moreover, since both systems have primary treatment, they should degrade approximately the same fraction of DDT to DDE—yet the DDE/DDT ratios differ dramatically. (ii) Currents that sweep materials north and west from the outfall, present in November 1970, swept the bulk of "fresh" sewage beyond the Palos Verdes Peninsula into Santa Monica Bay (*13, 15*). This "fresh" sewage would have a lower DDE/DDT ratio than older sewage so that animals exposed to it would contain relatively higher amounts of DDT. Low DDE/DDT ratios can be found near other locations where DDT entered the marine environment comparatively recently, such as mouths of rivers which drain major agricultural areas (points 1 and 7) and runoff from sites of local DDT usage (DDT was used on citrus trees near point 11 and presumably on Mexican crops near point 19).

Although *Emerita* near drainage from areas where DDT has been used show elevated concentrations of tDDT, the highest concentrations of tDDT in *Emerita* along the California coast are found near the effluent from the sewage system that accepts the industrial discharge from the plant where DDT is manufactured. This observation suggests that historically the buildup of residues in California coastal marine organisms could be attributed, to a significant degree, to industrial waste discharge rather than merely to extensive agricultural usage. Data taken at two points in time indicate that bottom sediments serve as an important reservoir for DDT residues from this input and that this reservoir may become available for biological uptake when these sediments are stirred up.

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Alkyl Isocyanates as Active Site-Specific Inhibitors of Chymotrypsin and Elastase

Abstract. *Alkyl isocyanates react specifically with the two serine proteinases, chymotrypsin and elastase, to yield inactive enzyme derivatives containing 1 mole of reagent per mole of enzyme. Octyl isocyanate inactivates chymotrypsin only, while butyl isocyanate inactivates both enzymes but shows greater efficiency toward elastase than toward chymotrypsin. These reagents may thus represent unique chemical "yardsticks" for the measurement of the relative dimensions of the active sites of the two very similar enzymes.*

Chymotrypsin and elastase are pancreatic proteinases with similar structure and catalytic function. The structural properties were first established by the complete elucidation of the primary amino acid sequences (*1*) and subsequently by high-resolution x-ray crystallography which has led to elegant three-dimensional models for both enzymes (*2*). The two molecules show extensive homology in their primary amino acid sequences and their folded structures are also remarkably similar (*3*). The active site serine (*4*), which is common to both enzymes (and which is acylated and deacylated in the amide and ester hydrolysis catalyzed by the enzymes), is located near the edge of a pronounced cavity or pocket on the surface of the two globular proteins. Since it has now been established in the case of chymotrypsin (*5*) that this cavity is responsible for the binding of the substrate component which contributes the acyl group to the amide or ester bond cleaved by the enzyme, the cavity has been referred to as the "binding pocket" (*3*). The only significant difference in the function of chymotrypsin and elastase is expressed by their sub-

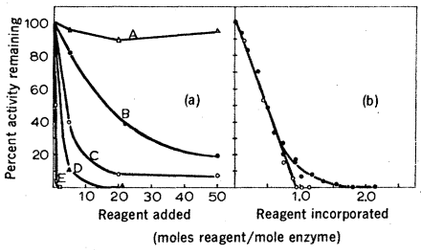

Fig. 1. (a) Inactivation of chymotrypsin and elastase as a function of alkyl isocyanate added. (Curve A) Elastase ($2 \times 10^{-6}M$) + OIC; (curve B) chymotrypsin ($2 \times 10^{-6}M$) + BIC; (curve C) elastase ($2 \times 10^{-6}M$) + BIC; (curve D) chymotrypsin ($2 \times 10^{-6}M$) + OIC; and (curve E) chymotrypsin ($8 \times 10^{-5}M$) + OIC. (b) Inactivation of chymotrypsin (●) and elastase (○) as a function of reagent incorporation. The concentration of both enzymes was $4 \times 10^{-4}M$. The relative specificity of BIC toward the two enzymes is reflected by the two inactivation curves and by the additional fact that 1.5 and 2.3 equivalents of BIC had to be added to elastase and chymotrypsin respectively to achieve 95 percent inhibition.

strate specificities, the former showing preference for aromatic and to a lesser degree for long-chain aliphatic residues while the latter is directed toward short-chain aliphatic residues, especially alanine. An examination of the detailed structure of the binding pockets of the two enzymes, as revealed by the x-ray crystallographic work, shows that the binding pocket of elastase is occluded by bulky amino acid residues replacing the less space-filling glycine residues in the corresponding positions in chymotrypsin, and it may thus be possible to explain the specificity of the two enzymes on the basis of the relative depth of their binding pockets (*3*). We present this report in support of such an explanation. The finding that two homologous reagents, octyl isocyanate (OIC) and butyl isocyanate (BIC), which differ only in the length of the aliphatic chain, give specific inactivation of chymotrypsin and elastase, respectively, is completely consistent with the predictions of the "binding pocket" hypothesis.

Chymotrypsin [Pentex, twice crystallized; about 85 percent pure by titration with transcinnamoyl imidazole (*6*)] and elastase (Worthington, electrophoretically pure) were reacted with the alkyl isocyanates in 0.1*M* tris HCl buffer (*p*H 7.6) at room temperature. An acetone solution of either OIC or BIC (both obtained from K and K Laboratories) was added to the rapidly stirring protein solution. The maximum acetone concentration in the reaction mixture was 2 percent (by volume). It is of interest to note that under these conditions both reagents hydrolyze readily, with a half-life of about 1 minute; the reaction with protein must therefore be quite selective in order to compete with the hydrolysis. At the protein concentrations used in the experiments reported here (2×10^{-6} to $4 \times 10^{-4}M$), the reaction with any portion of alkyl isocyanate was complete in less than 30 seconds; this finding is consistent with a facilitated reaction with the proper proteins. Enzyme activity was measured by direct rate assays with the use of casein and benzoyltyrosine ethyl ester (*7*) as substrates for chymotrypsin, and elastin and *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester (*8*) for elastase. For both enzymes, any loss of activity toward the large protein substrate was always parallel to that toward the small synthetic substrate.

The loss of chymotrypsin and elastase activity as a function of the molar equivalents of OIC and BIC added to the reaction mixture is shown in Fig. 1a. Octyl isocyanate does not react with elastase (curve A), but is a very effective inactivator of chymotrypsin (curve D); BIC, on the other hand, causes inactivation of both enzymes, but is more effective toward elastase (curve C) than toward chymotrypsin (curve B). These experiments were all conducted at protein concentrations of $2 \times 10^{-6}M$, and, in all cases of inactivation, a relatively large molar excess of reagent was required to titrate the total enzyme activity. As expected, however, increasing the enzyme concentration gave a more efficient inactivation reaction. At an $8 \times 10^{-5}M$ concentration of chymotrypsin, complete inactivation was obtained with only 1.5 moles of OIC added per mole of enzyme (curve E, Fig. 1a), and at $4 \times 10^{-4}M$ protein the reaction with BIC is very nearly stoichiometric for both enzymes (Fig. 1b).

In order to establish the precise stoichiometry of the reaction that leads to enzyme inactivation, the two enzymes were reacted with [^{14}C] BIC (New England Nuclear) labeled in the carbonyl carbon with ^{14}C. The results are given in Fig. 1b and show that the loss of activity is associated with the incorporation of exactly 1 mole of reagent per mole of enzyme.

These experiments demonstrate that OIC is a highly specific chymotrypsin reagent, and that BIC, although inactivating both enzymes, has a preference

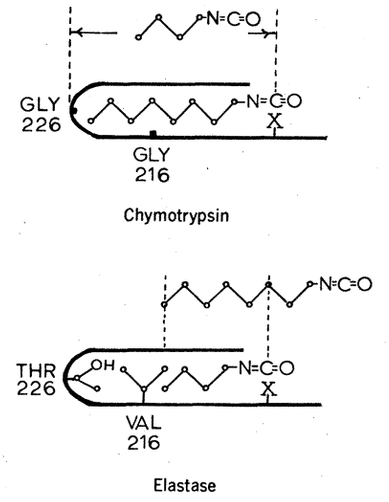

Fig. 2. Schematic representations of the binding pockets of chymotrypsin and elastase [based on Shotten and Watson's discussion (*3*)] and the possible alignment of the alkyl isocyanates in the binding pockets to give the enzyme-reagent complex (I) proposed as the obligatory precursor for the specific convalent bond formation (and inactivation).

for elastase. To fully document this specificity, it must be noted that under the conditions used for the experiments shown in curves A to D in Fig. 1a, other pancreatic enzymes, such as trypsin and carboxypeptidase A and B, were not affected by a 100-fold molar excess of OIC (*9*).

It is interesting to attempt to rationalize these findings in terms of the established structural features of chymotrypsin and elastase. We visualize the inactivation of both enzymes to proceed as follows:

$$E\text{–}XH + R\text{–}N{=}C{=}O \underset{}{\overset{1}{\rightleftharpoons}} \left[\begin{matrix} R\text{–}N{=}C{=}O \\ \vdots \\ E\text{——}XH \end{matrix}\right] \overset{2}{\longrightarrow} \begin{matrix} H \\ R\text{–}N\text{–}C{=}O \\ \vdots \quad | \\ E\text{——}X \end{matrix}$$

I

In the first step we propose that the enzyme forms a noncovalent complex (I) with the reagent, presumably based on high affinity of the alkyl side chain for the binding pocket. If this binding step leads to proper alignment of the two reactive groups (the isocyanate in the reagent and X in the enzyme), rapid covalent bond formation and inactivation should occur (step 2). We have no direct evidence for the existence of the noncovalent enzyme-reagent intermediate (I); yet without postulating such an intermediate it is difficult to explain both the discrimination exhibited by the two homologous reagents toward different proteinases

and the highly specific inactivation of the two enzymes in a process that must compete with both reagent hydrolysis and with nonspecific reactions at other reactive amino acid residues (*10*). This proposed reaction sequence, together with the very schematic and oversimplified representations of the binding pockets of chymotrypsin and elastase given in Fig. 2, provides a reasonable model for the inactivation of the two enzymes by alkyl isocyanates. According to this model, OIC is a specific reagent for chymotrypsin because this enzyme-reagent complex I gives perfect alignment of the reactive groups and therefore a high probability of covalent bond formation. In the BIC-chymotrypsin complex I, on the other hand, the alignment and therefore covalent bond formation is a less probable event. The binding pocket of elastase is very similar to that of chymotrypsin, but because of the presence of threonine and valine in positions occupied by glycine in chymotrypsin (*3*), the elastase binding pocket is shallower, and the model proposes that in this case only BIC can bind to give proper alignment for covalent bond formation. The longer OIC probably also binds, but because of the poor alignment, no reaction can occur between the isocyanate and X in the elastase-OIC complex I. At this stage the model in Fig. 2 is rather speculative. The reactive residue X has been placed in the position of the active site serine (residue 195 in chymotrypsin and residue 188 in elastase), and in analogy with the many other reactions by which this reactive serine residue can be derivatized (*11*), it is certainly the most likely site for the isocyanate reaction as well. In direct support of this proposition, we have isolated three, short, overlapping, radioactively labeled peptides after oxidation and proteolytic digestion of ^{14}C-labeled BIC-chymotrypsin by a procedure similar to that used by Shaw *et al.* (*11*). These peptides, comprising residues 193 to 199, 192 to 195, and 187 to 195, respectively, have residues 193 to 195 (glycine-aspartate-serine) as their common sequence, strongly suggesting that serine 195 indeed is the reactive residue in chymotrypsin. It can furthermore be concluded from the yield of radioactivity in each step of the peptide purification that this serine 195 derivative represents essentially all the incorporated isocyanate. This eliminates another possible reaction site, namely, histidine (*12*) at position 57. The final conclusions on this point must, however, await the results of similar studies on elastase. The ideal test for the model would be direct observation by x-ray diffraction analysis of the actual position of the alkyl chain in the two inactive enzyme derivatives.

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Signaled Reinforcing Brain Stimulation Facilitates Operant Behavior under Schedules of Intermittent Reinforcement

Abstract. *When single, rewarding brain stimulations were made predictable by preceding them with a brief warning signal, operant behavior was established and maintained under fixed ratio 200, variable ratio 30, fixed interval 3-minute, variable interval 2-minute, and differential reinforcement of low rate 20-second schedules of intermittent reinforcement. When the warning signal was removed, overall response rate declined in all but the fixed ratio schedules and then returned to the previous rate when the signal was reinstated.*

Brain stimulation reinforcement (BSR) has generally been less effective than food in establishing operant behavior under schedules of intermittent reinforcement. Sidman *et al.* (*1*) found that cats stopped responding when the ratio of responses to BSR's was greater than 8 : 1 [fixed ratio (FR) 8]. Similarly, behavior was not maintained when the value of a variable interval (VI) schedule exceeded 16 seconds, that is, when a response-contingent BSR was available only on the average of once per 16 seconds. The poor performance relative to that routinely maintained by conventional rewards such as food or water suggested to these authors that single BSR's were comparable to small amounts of food reward. Recently, Keesey and Goldstein (*2*) found that rats stopped responding when the schedule requirement of a progressive FR exceeded 30 : 1.

With the use of special procedures, performances like those usually obtained with food reinforcement have been obtained with schedules of intermittent BSR. Brown and Trowill (*3*), using rats, found that VI 1-minute and fixed interval (FI) 1-minute behavior could be maintained if the rat received not one but five response-contingent BSR's when a reinforcement was due. Pliskoff *et al.* (*4*) trained rats to perform the required schedule behavior (for example, VI or FR) on one lever to produce a second lever on which up to 20 BSR's were available on a continuous reinforcement schedule. In explanation of the typical performances that were obtained, the authors suggested (i) that the presentation of the second lever and the availability of continuous reinforcement simulated the early responses in the behavior chain leading to food, and (ii) that approximately ten BSR's were more similar to the amount of reinforcement in a standard food pellet than was one BSR.

The present data demonstrate that if a single BSR is made predictable by preceding it with a brief, exteroceptive warning signal, the establishment and maintenance of operant behavior under schedules of intermittent reinforcement is greatly facilitated.

Five male Sprague-Dawley rats, weighing approximately 300 g, were