olefins under conditions where the migration of the double bond occurs only to a small extent.[9]

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Received August 16, 1968

Enzymatic Displacement of Oxygen and Sulfur from Purines[1]

Sir:

Aminohydrolases have been found to catalyze hydrolytic removal of both nitrogen[2-6] and halogen substituents from the 6 position of purine derivatives. 6-Methoxypurine ribonucleoside was also found to be hydrolyzed to inosine,[3,4] but the position of bond cleavage was not determined. For these reasons, and because adenosine deaminase from takadiastase removes different substituents at similar limiting rates, hydrolysis *via* a purinyl–enzyme intermediate was proposed.[3,4] In contradication to this mechanism, an apparent exchange of radioactive hypoxanthine into 6-chloropurine was observed with bacterial adenine deaminase;[7] this was later found to be entirely due to a radioactive impurity.[8] Indeed the completely negative results obtained by these authors and ourselves[9] render an ammonia–enzyme or chloro–enzyme complex highly improbable.

We wish to report that adenosine deaminases from both takadiastase and calf duodenum, recently isolated as homogeneous proteins,[10] catalyze exchange of oxygen from $H_2{}^{18}O$ into inosine. Inosine is relatively stable,[11] partly due to tautomerization to the keto form,[12] so that exchange is rather slow and was not detected in earlier tests for rapid exchange.[13] Indeed exchange occurs so much more slowly than hydrolysis of methoxypurine ribonucleoside that it has also been possible to determine the position of bond cleavage of 6-methoxypurine ribonucleoside.

Samples containing 80 mg of inosine in 5 ml of 1.40 atom % excess $H_2{}^{18}O$, containing 0.1 *M* sodium acetate buffer, pH 5.4, were incubated at 25° with varying amounts of enzyme (units as described in ref 14) for 24 or 48 hr, along with controls containing no enzyme and controls containing adenosine and enzyme to provide fully labeled inosine after hydrolysis. The inosine concentration chosen in these experiments greatly exceeded its dissociation constant from either enzyme, as indicated by its K_i as an inhibitor of adenosine deamination.[14] Reaction was stopped and inosine was converted to hypoxanthine by heating samples for 1 hr at 100° in the presence of 0.3 *M* H_2SO_4. Adjustment to pH 5 with KOH precipitated hypoxanthine, which was washed with cold water, dried *in vacuo*, and converted to carbon dioxide by heating with mercuric chloride at 400° for 2 hr.[15] Excess HCl was removed by passing the pyrolysis products through a 7,8-benzoquinoline column. The oxygen-18 content of the carbon dioxide thus obtained was determined from the relative peak heights of the *m/e* 46 to 44 ratios on a CEC 21-614 residual gas analyzer, modified with an inlet for batch sample analysis.[16] The results, shown in Figure 1 and Table I, correspond to exchange rates lower by approximately five orders of magnitude than the limiting rate constants for adenosine deamination by the calf and takadiastase enzymes.

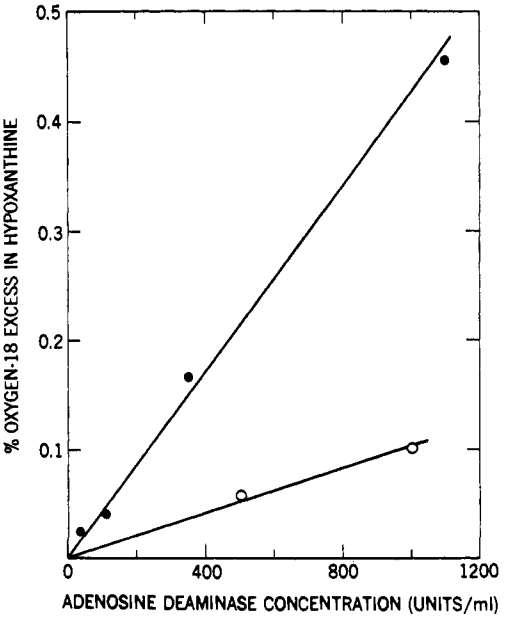

Figure 1. Per cent oxygen-18 in hypoxanthine recovered from inosine which had been incubated 24 hr with the indicated concentration of adenosine deaminase (corrected for nonenzymatic incorporation, 0.02% excess). Conditions: 80 mg of inosine, in 5.0 ml of 0.1 *M* sodium acetate buffer, pH 5.4, at 25°; 1.24% $H_2{}^{18}O$ excess; (●) enzyme prepared from calf intestine; (○) takadiastase enzyme.

In a second set of experiments, 6-methoxypurine ribonucleoside was hydrolyzed in $H_2{}^{18}O$ in the presence of the takadiastase enzyme (125 units/ml) for 4 hr at 25.[3] The resulting inosine was found to be within experimental error, fully labeled with ^{18}O. A parallel control containing inosine and enzyme underwent

(1) The work at Princeton University was supported by Research Grant GM-12725 from the National Institutes of Health, U. S. Public Health Service. The work at the University of California was supported by Research Grants GM-12278 from the National Institutes of Health, U. S. Public Health Service, and GB-4606 from the National Science Foundation.

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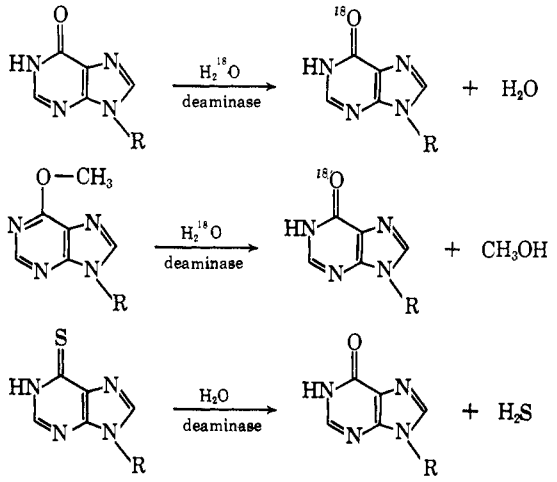

Figure 2. Reactions catalyzed by adenosine deaminase.

negligible exchange during the same period, as expected from the earlier results (Figure 1). Enzymatic cleavage of 6-methoxypurine ribonucleoside therefore occurs between C_6 and oxygen.

Table I. Rate Constants for Adenosine Deaminase Catalyzed Oxygen-18 Exchange with Inosine

Source of enzyme	k(ml/unit/hr) × 10^5
Calf intestine	1.76[a]
Takadiastase	0.41[b]

[a] Calculated from a first-order plot according to the equation

$$2.3 \log \frac{I}{I_\infty - I_E} = k[E]t$$

where I_∞ is the oxygen-18 excess in the completely exchanged product and I_E, the excess in product isolated after incubation with the corresponding concentration of enzyme, [E], for time, t = 24 hr. I_∞ and I_E were corrected for the small nonenzymatic exchange. [b] Calculated from the rate constant for the calf enzyme and the relative slopes of initial velocities shown in Figure 1.

Finally, 6-mercaptopurine ribonucleoside, itself completely stable in neutral solution, was found to be hydrolyzed to inosine and hydrogen sulfide in the presence of calf duodenal adenosine deaminase, as shown by spectrophotometry, paper chromatography, and analysis for sulfide. The binding constant of this substrate has not been determined, but the observed rate constant for this reaction, with 3.3×10^{-3} M substrate in 0.1 M potassium phosphate buffer, pH 6.5, was approximately 0.8 ml/unit hr, somewhat similar to the rate constant for oxygen exchange into inosine.

These reactions (Figure 2) illustrate the catalytic versatility of adenosine deaminases and are consistent with the purinyl–enzyme hypothesis for which independent kinetic support will be published elsewhere. The slowness of removal of the new leaving groups oxygen and sulfur presumably reflects, among other factors, the greater stability of C=O and C=S bonds than the C—N bond which is broken in the normal substrate adenosine.

Desulfuration of 6-mercaptopurine ribonucleoside is of interest for chemotherapy, since this is one of the most effective antitumor agents known,[17] and similar enzymes occur in mammalian serum, spleen, and intestine.[10] Oxygen exchange into inosine suggests a possible method of assay for the hypothetical deaminase which introduces inosinic acid residues at specific positions in yeast transfer RNA;[18–20] takadiastase adenosine deaminase has already been shown to deaminate adenylic acid residues in oligonucleotides.[14] It appears likely that any enzyme which catalyzes deamination of adenylic acid residues in a precursor of transfer RNA should also catalyze oxygen exchange into inosinic acid residues of the finished product.

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Received August 29, 1968

The Direct Observation of an Acyl-Enzyme Intermediate in the α-Chymotrypsin-Catalyzed Hydrolysis of a Specific Substrate at Neutral pH

Sir:

The hypothesis that chymotrypsin-catalyzed reactions involve an intermediate acyl-enzyme (eq 1) has proved very powerful in explaining the large body of data available for this enzyme.[1] The acyl-enzyme scheme predicts that the observed turnover kinetics should obey the equations derived from the Michaelis–Menten scheme (eq 2) and it also predicts quantitative

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \quad (1)$$
$$\qquad\qquad\qquad\qquad\qquad\qquad + P_1$$

$$E + S \overset{K_M}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P_1 + P_2 \quad (2)$$

relations between the macroscopic constants of this scheme and the microscopic constants of eq 1. Such

$$k_{cat} = k_2 k_3/(k_2 + k_3) \quad (3)$$

predictions have been verified for some nonspecific substrates (*e.g.*, *p*-nitrophenyl acetate[2] and methyl cinnamate[3]) by direct measurement of the individual rate constants, but direct tests of the scheme's applicability to specific substrates are difficult because of the high rates and the low concentrations of intermediates present in the turnover reaction. However, since the step in which an enzyme–substrate complex (ES) is converted to a covalent compound (ES′) is a nucleophilic reaction and since free carboxylic acids are reactive toward nucleophiles, the hypothesis implies that at low pH the acid corresponding to a specific ester or amide substrate will react with the enzyme to form an equilibrium mixture in which acyl-enzyme will be present (eq 4). The discovery that mixing chymotrypsin

$$E + AH \overset{K_p}{\rightleftharpoons} E \cdot AH \underset{k_3}{\overset{k_{-s}}{\rightleftharpoons}} ES' \quad (4)$$

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