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# TWO DIFFERENT HEPATIC ADENOSINE DEAMINASES IN THE CHICKEN

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SUMMARY

1 Sephadex chromatography of chicken liver preparations have revealed the presence of a large (110 000 mol. wt.) and a small (30 000 mol wt ) adenosine deaminase (adenosine aminohydrolase, EC 3 5 4 4)

2 The small hepatic enzyme (adenosine deaminase II) has been shown to be identical with the duodenal enzyme in every property compared These properties included sizes (by Sephadex chromatography), activation energies, substrate specificities, Michaelis constants and behavior with *p*-mercuribenzoate

3 The tissue-specific differences between hepatic and duodenal adenosine deaminases are due to the presence of the large enzyme (adenosine deaminase I) in the liver which is absent in the duodenum. This enzyme has an entirely different set of kinetic properties and does not show mercurial activation as do the duodenal enzyme and hepatic adenosine deaminase II

## INTRODUCTION

Adenosine deaminases (adenosine aminohydrolase EC, 3.5 4.4) have been studied from a wide variety of organisms Properties, such as relative substrate specificities[1–6], Michaelis constants[7–9], apparent energies of activation[8,9] and molecular weights[10,11] have been compared. BRADY[12] and BRADY AND O'DONOVAN[7,13], in their study of six mammalian species have found no significant differences in relative substrate specificities of adenosine deaminases from different tissues in the same animals, but differences were found between enzymes from different organisms MA AND FISHER[9] studied adenosine deaminases in a wide variety of vertebrates and found that high activation energies are characteristic of birds and that most amphibians have liver adenosine deaminases with low relative substrate specificities.

Several studies have been made of tissue-specific differences in adenosine deaminases FISHER, MA AND CHILSON[8] have reported that chicken liver adenosine deaminase is different from the chicken duodenal enzyme CHILSON AND KAPLAN (personal communication) and MA AND FISHER (unpublished results) have found a realtively large adenosine deaminase in frog livers (molecular weight estimated to be

in the range of 100 000) which has a much lower relative substrate specificity than the frog duodenal enzyme Recently ROCKWELL AND MAGUIRE[14] prepared an enzyme from ox heart which showed properties different from the calf intestinal enzyme. PFROGNER[15] has studied an adenosine deaminase from calf spleen which also is different from the enzyme in calf intestine

Results obtained in this present study show that the tissue-specific differences in adenosine deaminases from chicken liver and duodenum are due to the presence of an enzyme in the liver which is absent in the intestine There are two distinctly different enzymes in the liver (designated I and II) Enzyme II behaves exactly the same as the duodenal enzyme, and is entirely different from enzyme I. Differences in sizes, Michaelis constants, relative substrate specificities and activation energies are reported Chicken duodenal adenosine deaminase is unique among adenosine deaminases in that it is activated by mercurials[11] All other studies show only inhibition with *p*-mercuribenzoate[15–20] When chicken hepatic enzymes were treated with this sulfhydryl reagent, enzyme II was activated and enzyme I was inhibited These results strongly suggest that enzyme II is identical with the duodenal enzyme

## MATERIALS AND METHODS

Adenosine and deoxyadenosine were obtained from Calbiochem, 6-chloropurine riboside and 2,6-diaminopurine riboside were bought from K and K Laboratories Inc , *p*-mercuribenzoate and cordycepin (3-deoxyadenosine) were purchased from Sigma and Sephadex G-150 and blue dextran sulfate were obtained from Pharmacia Fresh chicken livers were used for all enzyme preparations. Glass-distilled water was used in all experiments.

### *Determination of enzyme activity*

Enzyme activity was measured by following the decrease in absorbance at 265 m$\mu$ (ref 21) A unit of enzyme activity is defined as that amount of enzyme which catalyzes the hydrolysis of 1 $\mu$mole of adenosine/min under standard conditions, *i e.*, with a 1-cm path-length cell containing 3.0 ml of 1 0 $10^{-4}$ M adenosine in 0 20 M potassium phosphate buffer (pH 7 0) at 38° Measurements were made with a model 2000 Gilford spectrophotometer Temperature was controlled by circulating water through the cell compartment

### *Preparation of the enzyme*

*Step 1. Ammonium sulfate fractionation.* Fresh chicken livers were homogenized in glass-distilled water at a concentration of 1 g of tissue with 5 ml of water The original extract was centrifuged at 18 000 × *g* in a refrigerated centrifuge for 1 h The supernatant was poured through a fine nylon net into a graduated cylinder to remove the floating thin layer of lipid material. The supernatant was brought to 45% saturation with solid $(NH_4)_2SO_4$ [22]. Slow addition and constant stirring are important After stirring at 4° for at least 2 h, the solution was centrifuged at 18 000 × *g* for 30 min and the sediment was discarded Sufficient $(NH_4)_2SO_4$ was added to the supernatant to give 65% saturation and the solution was maintained for several hours at 4° with constant stirring After centrifugation, the supernatant was discarded and the sediment was dissolved in a small amount of 0 05 M phosphate buffer (pH 7 0) The solution

was centrifuged to remove some insoluble material and then chromatographed on a Sephadex G-150 column

*Step 2. Gel filtration* Sephadex G-150 was sieved so that the particle size was larger than 88 $\mu$, and was allowed to swell in 0 05 M phosphate buffer for 6 h in a boiling water bath. The swollen gel was cooled and a column (1.6 cm $\times$ 81 cm) was poured in the cold and equilibrated overnight with flowing buffer. The void volume was determined with blue dextran. In all experiments a 1.0–1.5-ml sample was applied to the column and 2-ml fractions were collected. It was found that flow rate had no significant effect on the resolving power of this column.

*Step 3. Recovery of enzyme from the chromatography fractions* The chromatographic pattern showed two peaks of enzyme activity. Fractions from the leading edge of the first peak were pooled (see Fig. 1) Enough solid $(NH_4)_2SO_4$ was added to give 90% saturation After centrifugation, the sediment was suspended in a very small amount of 0.05 M phosphate buffer (pH 7 0) and recentrifuged The inactive supernatant was discarded The sediment was dissolved in a slightly larger volume of buffer. This solution was used to establish the properties of adenosine deaminase I The combined fractions of the trailing edge of the second peak (see Fig 1) were treated in the same way and the enzyme so recovered was used to establish the properties of adenosine deaminase II

Typically these procedures give a 50% recovery of total activity and at least a 10-fold increase in specific activity.

## RESULTS

### *Resolution of two adenosine deaminases*

When an active liver fraction obtained by ammonium sulfate fractionation is passed through a Sephadex G-150 column, it always shows a two-peak pattern as illustrated in Fig 1 This indicates that there are at least two adenosine deaminases present. Using the procedure described by ANDREWS[10], it was estimated that enzymes I and II have molecular weights of about 110 000 and 30 000, respectively The size of enzyme II is essentially the same as the duodenal enzyme[11].

### *Temperature studies*

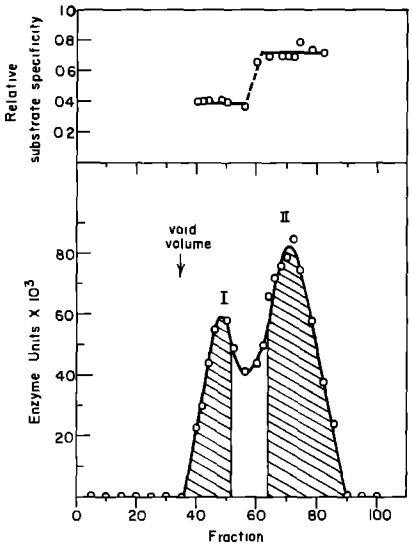
FISHER, MA AND CHILSON[8] reported an energy of activation ($E_a$) of 10 $\pm$ 2.2* kcal for chicken liver adenosine deaminase and a value of 15 $\pm$ 2 3 for the duodenal enzyme Results obtained with enzymes I and II show energies of activation of 4 $\pm$ 1 and 17 $\pm$ 2 kcal for enzymes I and II, respectively. The results of a typical experiment are shown in Fig. 2.

These results show that the energy of activation observed with liver preparations represents an average of the two very different values for enzymes I and II Enzyme II gives a result very close to that obtained previously with duodenal enzyme[1] and is in excellent agreement with a more recent study[11]

### *Relative substrate specificities*

A variety of substrates have been used in studies of chicken duodenal adenosine

* This represents the range of values obtained.



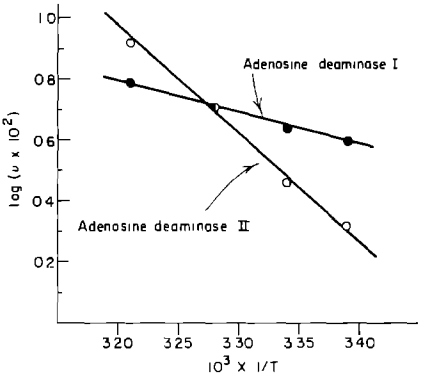




Fig 1 Resolution of two adenosine deaminases on a Sephadex G-150 column (1 6 cm × 81 cm) in 0 05 M potassium phosphate buffer (pH 7 0) Relative substrate specificity (ratio of enzyme activity with deoxyadenosine to that with adenosine) is shown as a guide for pooling fractions (2 ml each) Fractions from the shaded areas were pooled Void volume was determined with blue dextran

Fig 2 An Arrhenius plot of log $v$ *versus* the reciprocal of temperature in °K for chicken liver adenosine deaminase I (●—●) and II (○—○) Temperatures ranging from 23° to 38° were used. The adenosine concentration was 1 0 $10^{-4}$ M in 0 2 M phosphate buffer (pH 7 0) $v$ represents μM per min

deaminase It was of interest to compare the two adenosine deaminases resolved from chicken liver with the duodenal enzyme Activity was measured at 265 mμ when adenosine, deoxyadenosine and cordycepin (3-deoxyadenosine) were used as substrates[2-4,6] Dechlorination of 6-chloropurine riboside was followed at 250 mμ[2,5] and deamination of 2,6-diaminopurine riboside was followed at 247 mμ[1]. Chicken liver adenosine deaminase was previously reported to give a relative substrate specificity

TABLE I

RELATIVE SUBSTRATE SPECIFICITIES OF HEPATIC ADENOSINE DEAMINASES

All substrates were used at a concentration of 1 0 $10^{-4}$ M

| *Substrate* | *Chicken liver* | | | *Chicken duodenal adenosine deaminase*** |
|---|---|---|---|---|
| | *Adenosine deaminase I* | *Adenosine deaminase II* | *Adenosine deaminase** | |
| Adenosine | 1 00 | 1 00 | 1 00 | 1 00 |
| Deoxyadenosine | 0 36 | 0 71 | 0 63 | 0 74 |
| Cordycepin | 0 48 | 2 44 | — | 2 71 |
| 6-Chloropurine riboside | 0 11 | 0 43 | — | 0 51 |
| 2,6-Diaminopurine riboside | 0 05 | 0 48 | — | 0 61 |

* From FISHER, MA AND CHILSON[8]
** From HOAGLAND AND FISHER[11]

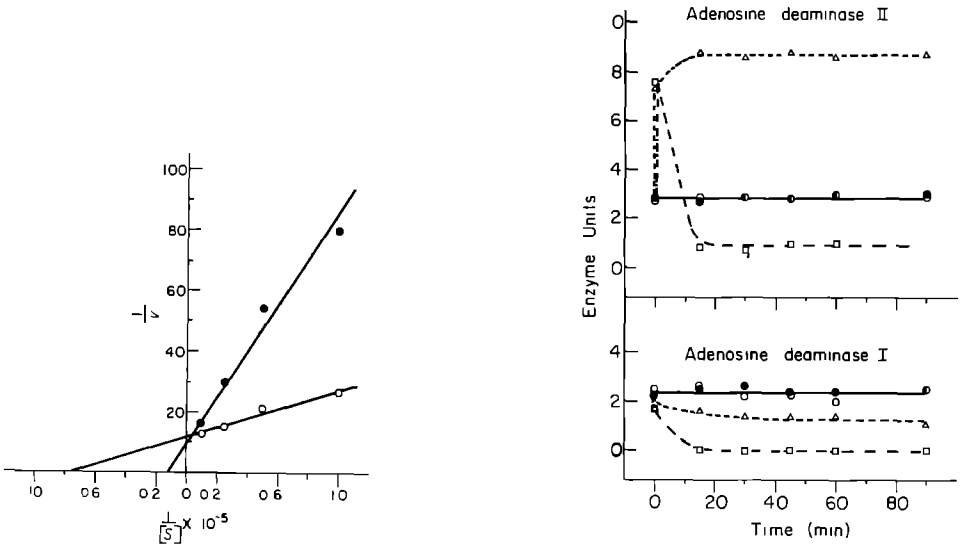



Fig 3 Lineweaver–Burk plots of chicken liver adenosine deaminase I (●—●) and II (○—○) $v$ represents $\Delta A$ per min and the concentration of substrate is in moles/l

Fig 4 Effects of *p*-mercuribenzoate on activities of chicken liver adenosine deaminase I (bottom figure) and II (upper figure) The different concentrations of *p*-mercuribenzoate used were 0 5 $10^{-5}$ M (○—○), 4 3 $10^{-5}$ M (△—△) and 8 6 $10^{-5}$ M (□—□) Phosphate buffer (0 05 M, pH 7 0) was used as the control (●—●) In each case equal volumes of enzyme solution and *p*-mercuribenzoate solution were mixed to give the final *p*-mercuribenzoate concentration indicated and the mixture was allowed to incubate at room temperature Aliquots were withdrawn at intervals and assayed for enzyme activity under standard conditions In these studies pooled fractions from Sephadex columns were used without concentration

(ratio of activity with deoxyadenosine to that with adenosine) of 0 63 Enzyme I gave a value of 0 36 and enzyme II a value of 0.72 (see Table I) This indicates that the previously obtained value represents an average of these two values when it is realized that different preparations may have different relative amounts of the two enzymes. Results presented in Table I show that enzyme II behaves essentially the same as the duodenal enzyme Enzyme I exhibits a completely different pattern.

*Michaelis constants*

The Michaelis constant of adenosine deaminase I is greater than that of adenosine deaminase II by a factor of seven. The previously reported value[8] for chicken liver enzyme was $3.3 \cdot 10^{-5}$ M In the present study, the $K_m$ values of chicken liver enzymes I and II are 9.2 ($\pm$1 0) $\cdot 10^{-5}$ and 1.3 ($\pm$0.2) $10^{-5}$ M, respectively. Results given in Fig 3 show the sharp contrasts in substrate dependence between these two enzymes The previously reported value of $3.3 \cdot 10^{-5}$ M represents an average of the Michaelis constants for these two enzymes HOAGLAND AND FISHER[11] reported a $K_m$ value of $1.3 \cdot 10^{-5}$ M for the chicken duodenal enzyme which is in excellent agreement with that for enzyme II.

*Effects of p-mercuribenzoate*

Chicken duodenal adenosine deaminase exhibits the very unusual property of

being activated by *p*-mercuribenzoate at the proper concentration[11] Since chicken liver adenosine deaminase II exhibited kinetic properties very much like the duodenal enzyme, it was expected that *p*-mercuribenzoate would also activate this enzyme. Results given in Fig 4 show that enzyme II can be converted to a stable active form and that higher concentrations of *p*-mercuribenzoate give a transient activation followed by inactivation These characteristics are exactly like those found for the duodenal enzyme[11] The fact that higher concentrations of *p*-mercuribenzoate were required in the present study probably only reflects the presence of other sulfhydryl-reacting substances in these relatively crude preparations

Enzyme I from chicken liver shows only inhibition or no effect if very low concentrations of *p*-mercuribenzoate are used. This is typical of results obtained with adenosine deaminases from most sources[15–20]

## DISCUSSION

In a previous study of chicken liver adenosine deaminase it was concluded that the liver and duodenal enzymes were different. Because frog liver was shown to contain a relatively large adenosine deaminase, it seemed possible that the liver of chickens also contains a large enzyme with different properties Sephadex chromatography revealed the presence of two adenosine deaminases in this tissue The smaller of the two enzymes (enzyme II) was found to be strikingly similar to the duodenal enzyme They exhibit the same activation energies, Michaelis constants, and the same relative velocities with a variety of substrates including one which is dechlorinated The very unusual behavior of the duodenal enzyme in the presence of mercurials is also exhibited by liver adenosine deaminase II This involves formation of a stable derivative with two to three times more activity than the native enzyme at relatively low concentrations of the mercurial At higher concentrations transient activation is observed, followed by inactivation

Liver adenosine deaminase I has properties markedly distinct from liver enzyme II and the duodenal enzyme It exhibits an activation energy of 4 kcal (compared to 17 for enzyme II), a Michaelis constant of $9.2 \cdot 10^{-5}$ (compared to $1.3 \cdot 10^{-5}$) and a pattern of relative substrate specificities entirely different from liver enzyme II. Furthermore, *p*-mercuribenzoate simply inhibits activity in the manner typical of all adenosine deaminases which have been studied, except for the chicken duodenal enzyme and hepatic enzyme II.

On the basis of these results it is clear that the tissue specific differences between liver and duodenum in the chicken are due to the presence of an enzyme in the liver which is not present in the duodenum. However, there are enzymes in these tissues which are identical in every property compared

The presence of adenosine deaminases of different sizes in the chicken liver and in the frog liver suggests that such multiple forms may be found in many vertebrates. MA AND FISHER (unpublished) have examined hepatic adenosine deaminases in a wide variety of fish, amphibians, reptiles, birds and mammals in an effort to determine whether or not this pattern is common Multiple adenosine deaminases of different sizes have been found in all vertebrate classes, including mammals Generally they fall into three classes by size Most have molecular weights in the order of 30 000, numerous examples are in the order of 100 000 and one has been found with an esti-

mated molecular weight of 150 000–200 000 On the basis of these results, it seems clear that vertebrate adenosine deaminases exhibit an unusual diversity in size.

This variability raises the question of homology, *i e*, have all of these enzymes arisen from a common ancestral type? At the present time it is not possible to answer this question with any confidence. Detailed studies of one or several of the larger enzymes will be necessary. So far only adenosine deaminases in the size range of 30 000 have been studied in any detail.

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