Studies on the Mechanism of the Enzymatic Amination and Hydration of Fumarate*

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The finding that the hydration of fumarate in deuterium oxide, as catalyzed by pig heart fumarase, resulted in monodeuterio-l-malate, and that prolonged incubations did not lead to any appreciable isotope incorporation into fumarate, established the stereospecific nature of this reaction (1-3). Through the application of the proton magnetic resonance solid state method, Farrar et al. (4) showed that the protons on carbons 2 and 3 in the monodeuterio-l-malate are gauche to one another. On the assumption that the carboxyl groups in crystalline malic acid are trans, the conclusion was reached that fumarase catalyzes a cis addition to the double bond of fumarate.

Studies relating to the stereospecificity of enzymatically catalyzed saturation reactions have been restricted to the addition of water to carbon-carbon double bonds (1-3, 5). It was therefore of interest to study reactions in which groups other than water were added to carbon-carbon double bonds. Accordingly, a study was undertaken to delineate some features of the aspartase reaction. This enzyme, catalyzing the reversible deamination of aspartic acid, has been described in a wide variety of microorganisms and its preparation from Bacterium cadaveris in partially purified form has recently been reported (6).

The results presented in this paper show that aspartase catalyzes a stereospecific addition of ammonia to the double bond of fumarate. In addition, evidence is presented suggesting that both the aspartase and fumarase from B. cadaveris catalyze a cis addition of ammonia and water, respectively, to the double bond of fumarate.

ANALYTICAL METHODS AND MATERIALS

The aspartase used in the experiment described in Table I was a partially purified preparation from B. cadaveris prepared according to the method described by Williams and McIntyre (6) and then lyophilized. This preparation was practically devoid of fumarase activity since on deamination of L-aspartate good agreement was obtained between the amounts of fumarate and ammonia produced. The other experiments were conducted with preparations consisting of 20 to 50 per cent ammonium sulfate fraction from a sonic extract of an 18 hour culture of B. cadaveris. These latter preparations, which were dialyzed before lyophilization, were contaminated with fumarase to varying degrees. All these preparations were supplied through the generosity of Dr. Virginia R. Williams.

Aspartase activity was determined by measuring the extent of aspartate deamination, either through analysis of the liberated ammonia by nesslerization or by the spectrophotometric estimation of fumarate in those preparations essentially freed of fumarase activity. Crystalline pig heart fumarase was prepared by the method of Massey (7) and its activity was determined by measuring the rate of decrease in optical density at 240 or 300 mp due to fumarate hydration (8). Malic dehydrogenase was prepared by the procedure outlined by Ochoa (9). The aspartase-α-ketoglutarate transaminase was a crude, dialyzed, lyophilized extract from minced pig heart prepared by the method of Nisonoff et al. (10). This preparation contained 2.7 units of fumarase activity and 3800 units of malic dehydrogenase activity per mg of freshly prepared powder, as determined by the conventional assay procedures for these enzymes (8, 9). Transaminase activity was determined by the rate of decrease in optical density at 340 mp of a reaction mixture containing 150 μmoles of potassium phosphate, pH 7.3, 0.3 μmoles of DPNH, 10 μmoles of α-ketoglutarate, 20 μmoles of L-aspartate, and excess malic dehydrogenase, in a total volume of 3.0 ml. A unit of transaminase activity represents the amount of enzyme that causes a decrease in absorption at 340 mp of 0.001 per minute by use of the assay conditions described. DPNH was prepared enzymatically (11) with the use of crystalline yeast alcohol dehydrogenase (Mann Research Laboratories). α-Ketoglutaric acid was purchased from the California Foundation for Biochemical Research.

The various reaction mixtures, as well as the details of the diverse experimental procedures, are described in the text or in the legends of the tables. In each experiment the reaction was stopped either by the addition of perchloric acid or by heating for a short period of time. The deproteinized solutions were adjusted with KOH to pH 7.5 to 8.5 and passed through Dowex 1-formate (8 per cent cross-linkage) columns. The acids were separated by elution with continually increasing formic acid concentrations as described by Busch et al. (12). After desiccation, the fractions representing individual peaks were combined by solution in H2O and taken to dryness by flash evaporation. Identification of each peak was established by its position of emergence from the Dowex 1-formate columns as determined by titration with NaOH after desiccation. In addition, the identity of the malic and fumaric acid samples was determined by paper chromatography as previously described (3). The identity of

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the aspartic acid peak was further established by paper chromatography in the phenol-H$_2$O system described by Consden et al. (13).

Before the isotope was diluted by addition of carrier, as was done in some experiments, the various acids were determined quantitatively by the following methods. Aspartic acid was estimated by the ninhydrin method as modified by Moore and Stein (14). Malic acid was measured fluorometrically by the method of Speck as described by Loewus et al. (15), or spectrophotometrically by the method of Goodban and Stark (16). Fumaric acid was estimated by measuring the optical density at 240 m$\mu$ (8). Aspartic and fumaric acids were finally isolated as the free acids after several recrystallizations from hot water. Malic acid was isolated as the diphenacyl ester (15) and recrystallized two to three times from benzene and petroleum ether.

Throughout the isolation and purification procedures described for each acid, there was ample opportunity to wash out by exchange any unstably bound deuterium. After prolonged desiccation over P$_2$O$_5$ the samples, on combustion, yielded water for each acid, there was ample opportunity to wash out by exchange any unstably bound deuterium atom per molecule (Reaction 1). Hence, the reaction when carried out in D$_2$O can never lead to labeled fumarate or to L-aspartate containing more than 1 stably bound deuterium atom per molecule (Reaction 1).

\[
\begin{align*}
\text{COOH} & \quad \text{COOH} \\
\text{C-H} + \text{NH}_3 & \quad \text{D-C-H} \\
\text{H-C} & \quad \text{H-N-C-H} \\
\text{COOH} & \quad \text{COOH}
\end{align*}
\]

If the reaction, however, were nonstereospecific, deamination would lead to dideuterated fumarate, which could in turn lead to L-aspartate containing 2 stably bound deuterium atoms. Such a dideuterated L-aspartate molecule could lead to dideuterated fumarate, and hence on full isotopic equilibrium a nonstereospecific mechanism could give rise to L-aspartate containing a maximum of 3 stably bound deuterium atoms per molecule.

The results of an experiment outlined in Table I indicate that, whereas incubation of a mixture of L-aspartate, fumarate, and ammonia with aspartase in heavy water led to a continual high level of deuterium incorporation into L-aspartate, the deuterium content of the reisolated residual fumarate was extremely low. Under the conditions of this experiment there was a gradual conversion of fumarate to L-aspartate throughout the incubation period and neither chemical nor isotopic equilibrium was fully achieved. After dilution, the 10 hour deutero-L-aspartate sample was subjected to the further action of aspartase in normal water with the results outlined in Table II. This preparation of aspartase was heavily contaminated with fumarase so that the fumarate arising by deamination of the L-aspartate was rapidly hydrated to L-malate. Since the fumarase from B. cadaveris was later shown to behave stereospecifically, the extent of labeling in malate reflects the isotopic content of the fumarate arising by the enzymatic deamination of L-aspartate.

It is evident from the data summarized in Table II that the deuterium content of the diphenacyl malate derivative and therefore of the original fumarate, if significant at all, is exceedingly low. The residual reisolated aspartate in this experiment, because of the reversibility of the reaction as carried out in normal water, lost 92 per cent of its initial deuterium content in the course of the extended incubation period. The data presented in Table III show that the L-aspartate, reisolated from a reaction which proceeded for a period of time after the aspartase equilibrium had been established, contained no more than 1 atom of deuterium per molecule. These results show unequivocally that aspartase catalyzes a stereospecific proton addition to form the methylene group of L-aspartate according to Reaction 1. The extent of labeling found in the fumarate samples (as well as in the malate sample referred to in Table II), although low, appeared to be significant and seemed to bear a direct relationship to the period of the incubation. In view of the demonstrated stereospecificity of the aspartase reaction and an analogous behavior of the contaminating fumarase (see

### Table I

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Fumaric acid disappearing from reaction mixture</th>
<th>Aspartic acid</th>
<th>Fumaric acid</th>
<th>Deuterium content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aspartic</td>
<td>Fumaric</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acid</td>
<td>Acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Excess</td>
<td>Mole</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.523</td>
<td>6.08</td>
<td>0.426</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.238</td>
<td>0.69</td>
<td>0.853</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4.720</td>
<td>0.438</td>
<td>1.41 X 10^-3</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>5.664</td>
<td>0.69</td>
<td>0.72 X 10^-3</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>5.897</td>
<td>10.28</td>
<td>0.719</td>
<td></td>
</tr>
</tbody>
</table>

* The presence of 1 atom of deuterium per molecule of aspartic acid and fumaric acid would correspond to values of 14.29 and 25.0 atom per cent excess, respectively.
intervals, 0.1 ml. aliquots were added to 4.9 ml. of 0.33 N perchloric acid; NH₄⁺ and fumarate were then determined on 0.1 ml. aliquots of the supernatant. It was thus established that after 1 hour 73.1 per cent of the total NH₃ that was liberated relative to the 4 hour sample was accounted for. At 4 hours 25 ml. of the reaction mixture were added to 3.2 ml. of 1.65 N perchloric acid, and the protein residue was washed once with 3 ml. of 0.187 N perchloric acid and discarded. Aspartic and malic acids were isolated from the combined supernatants and analyzed for deuterium content.

Stereospecific Behavior of B. cadaveris Fumarase—In order to assess whether a stereospecific mechanism also determined the course of the bacterial fumarase reaction, L-malate, arising from some of the present experiments as a result of the contaminating fumarase activity, was isolated and its deuterium content was determined. The results of an experiment outlined in Table III indicate that whereas the malate exhibited high deuterium content, as anticipated, it did not exceed the theoretical value of 1 atom of deuterium per molecule inherent in a stereospecific mechanism of saturation. The reaction proceeded significantly after the fumarase (and aspartase) equilibria had been reached, so that there was ample time for the fumarase to become more highly labeled and for the L-malate (and the L-aspartate) to incorporate more than 1 atom of deuterium per molecule if the fumarase (and/or the aspartase) behaved in a nonstereospecific manner.

Large Scale Preparation of Monodeutero-L-Aspartic Acid—19.90 mmoles of fumaric acid, 41.35 mmoles of NH₄Cl, 10.25 mmoles of potassium phosphate, and 204.7 mg. of a lyophilized aspartase preparation in a total volume of 205 ml. of 0.020 N perchloric acid was adjusted to pH 8.2, as measured with glass electrodes, was incubated for 6 hours at 34-35°. At various time intervals, 0.1 ml. aliquots were added to 4.9 ml. of 0.33 N perchloric acid; NH₄⁺ and fumarate were then determined on 0.1 ml. aliquots of the supernatant. It was thus established that after 1 hour 73.1 per cent of the total NH₃ that was liberated relative to the 4 hour sample was accounted for. At 4 hours 25 ml. of the reaction mixture were added to 3.2 ml. of 1.65 N perchloric acid, and the protein residue was washed once with 3 ml. of 0.187 N perchloric acid and discarded. Aspartic and malic acids were isolated from the combined supernatants and analyzed for deuterium content.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Acids Isolated</th>
<th>Deuterium content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Atom % excess*</td>
</tr>
<tr>
<td>No incubation</td>
<td>Aspartic</td>
<td>1.49</td>
</tr>
<tr>
<td>Enzyme incubation</td>
<td>Aspartic</td>
<td>0.122</td>
</tr>
<tr>
<td></td>
<td>Malic</td>
<td>0.020</td>
</tr>
</tbody>
</table>

* Experimental values.
† 1 atom of deuterium per molecule of aspartic acid and di-phenacyl malate would correspond to 14.29 and 5.55 atom per cent excess, respectively.

Effect of bacterial aspartase and fumarase on deuterium incorporation into aspartate, malate, and fumarate

The reaction mixture contained, in a total volume of 33.0 ml., 3.32 mmoles of fumarate, 5.88 mmoles of NH₄Cl, 1.65 mmoles of potassium phosphate and 50 mg. of aspartase powder (containing some fumarase). The D₂O concentration of the medium was 98 to 99 per cent and the pH was adjusted to 6.8, as measured with glass electrodes. The reaction was started by the addition of enzyme; incubation was conducted at 31°. At various time intervals over the 6 hour period, 0.1 ml. aliquots were removed and added to 4.9 ml. of 0.33 N perchloric acid. These were stored in an ice bath until all the samples were collected. Each sample was centrifuged and the supernatant fluids were assayed for residual NH₄Cl content by direct nesslerization and for residual fumarate by absorption at 240 mμ. It was thus established that the reaction had reached equilibrium at the end of 6.5 hours, at which time 2.77 mmoles of NH₄Cl and 3.01 mmoles of fumarate had disappeared. The difference between these values represents the amount of malate synthesized. At the indicated time intervals, 15 ml. aliquots were removed and the reaction stopped by heat inactivation. Aspartic, malic, and fumaric acids were isolated and analyzed for deuterium content.

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Acids isolated</th>
<th>Dilutions</th>
<th>Deuterium content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Atom % excess*</td>
<td>Atom/molecule</td>
</tr>
<tr>
<td>hrs.</td>
<td>Aspartic</td>
<td>15.5</td>
<td>0.071</td>
</tr>
<tr>
<td></td>
<td>Malic</td>
<td>10.0</td>
<td>0.511</td>
</tr>
<tr>
<td></td>
<td>Fumaric</td>
<td>2.02</td>
<td>0.007</td>
</tr>
<tr>
<td>6</td>
<td>Aspartic</td>
<td>9.43</td>
<td>1.528</td>
</tr>
<tr>
<td></td>
<td>Malic</td>
<td>10.1</td>
<td>0.525</td>
</tr>
<tr>
<td></td>
<td>Fumaric</td>
<td>5.74</td>
<td>0.029</td>
</tr>
</tbody>
</table>

* Experimental values.
† The values are corrected for dilution and calculated on the basis that 1 atom of deuterium per molecule of aspartate, di-phenacyl malate, and fumarate would correspond to values of 14.29, 5.55, and 25.0 atom per cent excess, respectively.

Chloric acid; NH₄Cl and fumarate were then determined on suitable aliquots of the deproteinated supernatant fluids. It was ascertained that in 6 hours, a total of 14.62 and 11.59 mmoles of fumarate and NH₄Cl, respectively, disappeared. The excess of fumarate lost during the course of the reaction could be accounted for as malate formed by the action of fumarase which was present in the aspartase preparation. The reaction was stopped by heat inactivation, and the deproteinized solution was reduced in volume by flash evaporation. After adjustment to pH 8.2 with KOH, the solution was passed through a Dowex 1-formate column with a large amount of water. Aspartic acid was eluted with 1.25 N formic acid. Subsequent to desiccation, the aspartic acid was recrystallized from hot water and dried over P₂O₅; the yield was 1.4774 gm. (11.09 mmoles). An average of three determinations on this sample indicated an atom per cent excess of deuterium of 13.47 corresponding to 0.943 atoms of deuterium per molecule of aspartic acid.

Chemical Conversion of Enzymatically Synthesized Monodeutero-L-Aspartic Acid to Deutero-L-Malic Acid—To 0.1313 gm. (1 mmoles) of monodeutero-L-aspartic acid (0.943 atoms of deuterium per molecule) in 5.0 ml. of 1 N H₂SO₄ was added 1.5 ml. of a 30 per cent NaNO₂ solution; the addition was made over a
period of 20 minutes with continuous agitation. This solution was stirred for 1 hour and 10 minutes more, after which time a quantitative ninhydrin test on an aliquot showed no measurable aspartic acid. The solution was further acidified with dilute H$_2$SO$_4$ to pH 1.5, mixed with Celite, and continuously extracted with ether for 9 hours. The ether-extracted material was dissolved in water, adjusted to pH 8.0 with KOH, and passed through a Dowex 1-formate column. Malic acid was eluted with continually increasing concentrations of formic acid. The material was rechromatographed to achieve further purification, yield, 0.578 mmoles. A deuterium analysis on a diphenacyl derivative of an aliquot of this sample revealed that the malic acid contained 0.989 atoms of deuterium per molecule and hence the nitrous acid deamination afforded a quantitative retention of the deuterium initially present on the methylene carbon of the monodeutero-L-aspartic acid.

Enzymatic Conversion of Enzymatically Synthesized Mono-
deuterated L-Aspartic Acid to Deutero L-Malic Acid—The reaction mixture consisted of 3.0 mmoles of potassium phosphate, 1.0 mmoles of monodeutero-L-aspartate (0.943 atoms of deuterium per molecule), 2.0 mmoles of $\alpha$-ketoglutarate, 0.505 mmoles of DPNH, 464,000 units of transaminase, and 297,400 units of malic dehydrogenase in a total volume of 60 ml., pH 7.3. The reaction was initiated by the addition of transaminase, and incubation was carried out at room temperature. At various time intervals, 0.05 ml aliquots were removed and rapidly added to 3.0 ml of 0.05 M phosphate buffer of pH 7.3, and the optical density at 340 m$\mu$ was measured immediately against 0.05 ml of a blank, containing all of the above components except DPNH, which was added to 3.0 ml of the same buffer. It was thus ascertained that nearly all of the added DPNH had been reoxidized within 3.5 minutes. After 9 minutes, the reaction was stopped by heat inactivation. More complete deproteinization was achieved by the addition of perchloric acid to a final concentration of 0.33 N. The protein-free solution was neutralized to pH 7.5 with KOH, and the potassium perchlorate removed by filtration. After reducing the volume by flash evaporation, the solution was acidified to pH 1.5 with dilute H$_2$SO$_4$, mixed with Celite, and continuously extracted with ether for 15 hours. The ether-extracted material that was dissolved in water and adjusted to pH 8.3 with KOH was chromatographed on a Dowex 1-formate column, separation of malic acid was achieved as before; yield, 0.364 mmoles. The diphenacyl derivative of an aliquot of this sample had 2.06 atom per cent excess deuterium corresponding to 0.371 atoms of deuterium per molecule of malic acid. In part, the 61 per cent loss of deuterium relative to the isotopic content of the starting monodeuterated aspartic acid occurs by way of the keto-enol tautomerization of the oxaloacetate acid which is an intermediate in the enzymatic transformation of aspartic acid to malic acid. Deuteration incorporation into malate by way of this nonenzymatic keto-enol tautomerism has previously been observed in studies designed to establish (a) whether the enol or keto form of oxaloacetate acid is involved in the reduction catalyzed by wheat germ malic dehydrogenase (15) or (b) which form arises from the action of spinach and wheat germ phosphoenolpyruvate carboxylase (17, 18) and from bird liver phosphoenolpyruvate carboxylase kinase (19). In addition, the transaminase preparation used in the present experiment contained a small amount of fumarase. In view of the subsequent demonstration that all the enzymes investigated in this study exhibited identical stereospecificity with respect to the methylene group hydrogen atoms, the possibility must be considered that part of the deuterium was washed out from the L-malate by way of the reaction catalyzed by fumarase.

Mechanism of Aspartase-Catalyzed Stereospecific Amination of Fumarate to L-Aspartate—Fumarase and aspartase show strict stereospecificity toward the L-optical isomers, and thus both enzymes exhibit identical stereospecificity with respect to the asymmetrical carbon atom of L-malate and L-aspartate, respectively. The finding that the enzymatic hydration and amination of fumarate in deuterium oxide yielded exclusively monodeutero-L-malate and monodeutero-L-aspartate, respectively, established the stereospecific nature of deuterium addition in the formation of the methylene group of both L-malate and L-aspartate. It was therefore of interest to establish whether fumarase and aspartase exhibited identical stereospecificity in the addition of deuterium on one of the C—H groups of fumarate.

The experimental approach rested on the following arguments. If the monodeutero-L-aspartate resulting from the aspartase reaction in a medium enriched with D$_2$O has the same configuration around the methylene carbon atom as the product of the mammalian fumarase reaction, then dehydration by fumarase of the L-malate obtained either chemically or enzymatically from monodeutero-L-aspartate, should yield essentially nondeuterated fumarate. On the other hand, if the position of the deuterium atom of the methylene group of the compounds under consideration is of opposite configuration, the resulting fumarate should retain all the deuterium initially present in the monodeutero-L-malate obtained from the enzymatically synthesized monodeutero-L-aspartate. The results of such an experiment are summarized in Table IV. It is apparent that the action of crystalline pig heart fumarase on the deutero-L-malate, obtained either chemically or enzymatically from monodeutero-L-aspartate, yielded fumaric acid with a low order of isotope content. The L-malate resolated from the reaction mixture as the diphenacyl derivative retained only 26 per cent of the initial deuterium content of the chemically formed L-malate and 19 per cent in the case of L-malate enzymatically synthesized. This was due to prolonged incubation beyond the time required for chemical equilibration and no doubt reflects a stereospecific washing out of the deuterium by the action of fumarase, which seems evident from the low level of labeling in the corresponding fumarate samples.

As can be noted, however, the experiments with chemically and enzymatically converted L-malate presented some quantitative differences. The residual deuterium content of the fumarate resulting from the action of fumarase on the chemically synthesized deutero-L-malate was 3.3 and 12.9 per cent of the initial deuterium content of the chemically formed $L$-malate and 19 per cent in the case of L-malate enzymatically synthesized. This was due to prolonged incubation beyond the time required for chemical equilibration and no doubt reflects a stereospecific washing out of the deuterium by the action of fumarase, which seems evident from the low level of labeling in the corresponding fumarate samples.
cause of the keto-enol tautomerization of the intermediate monodeutero-oxaloacetate. Such a mechanism would no doubt cause a partial racemization of the methylene-bound deuterium atoms in oxaloacetate and on further reduction with DPNH by malic dehydrogenase would result in the yield of a mixture of two species of monodeutero-L-malate with opposite configurations. It is therefore not surprising that deuter-L-malate, enzymatically produced, was transformed into fumarate with a higher degree of deuterium retention than when treated with fumarase. In any event, the results show that the monodeuterated products of the mammalian fumarase and bacterial aspartase reactions have the same configuration around the methylene group. Therefore, both enzymes catalyze an identical stereospecific deuterium addition in the course of saturating the double bond of fumarate.

Mechanism of Stereospecific Hydration of Fumarate to L-Malate Catalyzed by B. cadaveris Fumarase—The DPN dehydrogenases which catalyze a direct stereospecific transfer of hydrogen to the nicotinamide moiety of DPN⁺ fall into two classes, depending on the side of the nicotinamide ring to which the hydrogen is added (20). These enzymes therefore do not share an identical stereospecific mode of hydrogen transfer. Opposite stereospecific actions on carbon-bound hydrogens have also been recorded for phosphoglucose and phosphomannose isomerase acting on fructose-6-phosphate (21), and for muscle aldolase and triosephosphate isomerase acting on dihydroxyacetone phosphate (22). Similar considerations, applied to reactions involving additions across carbon-carbon unsaturations, do not justify the assumption that the stereospecific hydration of fumarate by the fumarase of B. cadaveris is identical with the hydration catalyzed by mammalian fumarase. Table V summarizes the data of an experiment carried out to determine whether the bacterial fumarase adds deuterium in the same position as pig heart fumarase. It is evident that the monodeutero-L-malate, obtained by incubating fumarase with bacterial fumarase in deuterium oxide, was transformed to unlabeled fumarate by crystalline pig heart fumarase; and that, as a result of the prolonged incubation, approximately two-thirds of the initial deuterium content of the L-malate was washed out. These results establish the configurational identity of the products of both enzymes, and point to an identical stereospecificity in their mode of action.

DISCUSSION

The findings that the amination and hydration of fumarate by the aspartase and fumarase respectively of B. cadaveris, as carried out in deuterium oxide, yielded exclusively monodeuterated products with little, if any, deuterium incorporation in the residual fumarate, demonstrate that the reactions occur in a stereospecific manner. The stereospecific behavior of these two enzymes is analogous to that reported for pig heart fumarase andaconitase (1-3, 5). In addition, a kinetic consideration of deuterium incorporation into L-aspartate in the experiment described in Table I suggests that an intermediate mechanism is involved in the aspartase reaction similar to that proposed for the fumarase (23) and aconitase (24, 5) reactions. In this experiment there was a continuing disappearance of fumarate throughout the incubation period. Assuming that the preparation of aspartase was but slightly contaminated with fumarase, since only traces of malate could be detected, it is likely that the fumarate which disappeared was almost quantitatively con-

**Table IV**

| Reaction of crystalline pig heart fumarase on deuter-L-malate derived from enzymatically synthesized monodeutero-L-aspartate
<table>
<thead>
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<tbody>
<tr>
<td><strong>Experiment</strong></td>
</tr>
<tr>
<td></td>
</tr>
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<td></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
</tbody>
</table>

*Experimental values.*

† The values were corrected for dilution (when applicable) and calculated on the basis that 1 atom of deuterium per molecule of diphenacyl malate and fumaric acid corresponds to values of 5.55 and 25.0 atom per cent excess, respectively.

‡ The samples were diluted 2.25- and 2.19-fold in Experiments 1 and 2, respectively.

**Table V**

| Reaction of crystalline pig heart fumarase on monodeutero-L-malate derived enzymatically by action of Bacterium cadaveris fumarase
<table>
<thead>
<tr>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial deuterium content of L-malate</strong></td>
</tr>
<tr>
<td><strong>Atom % excess</strong></td>
</tr>
<tr>
<td>L-malate</td>
</tr>
<tr>
<td>0.324</td>
</tr>
</tbody>
</table>

*Experimental values.*

† The values were corrected for dilution (when applicable) and calculated on the basis that 1 atom of deuterium per molecule of diphenacyl malate and fumaric acid corresponds to values of 5.55 and 25.0 atom per cent excess, respectively.

‡ The samples were diluted 2.40-fold.
verted into L-aspartate. Knowing the amount of unlabeled aspartate initially present in the reaction mixture, which would dilute the monodeutero-L-aspartate arising enzymatically from fumarate, one can calculate the theoretical minimal deuterium content of the aspartate at each time interval. Such calculations revealed values of 0.460, 0.645, 0.725, 0.760, and 0.768 atoms of deuterium per molecule of aspartate for the 2, 4, 6, 8, and 10 hour samples, respectively. The experimental values do not exceed, and indeed are actually lower than, the calculated levels of isotope incorporation, especially in the early samples. These results therefore support the view that little, if any, deuterium exchanges into the unlabeled aspartate, and that the bulk of the isotope detected in the aspartate is a result of a net synthesis from fumarate. Alberty et al. (23) have shown that the degree of deuterium incorporation into L-malate, on incubation with fumarase in D₂O, is in quantitative agreement with that expected from the over-all reversibility of the reaction. Similarly, in the case of aconitase (5) the incorporation of deuterium into citrate on incubation in a medium enriched with D₂O appears to occur via the process: citrate = cis-aconitate = labeled citrate. It therefore appears likely, in analogy to the fumarase and aconitase reactions, that the enzymatic deamination of aspartate proceeds via a carbonium ion intermediate devoid of an amino group rather than through a carbanion arising by the loss of a proton from the methylene group of aspartate.

The simple dehydration by pig heart fumarase of the monodeutero-L-malate synthesized by the bacterial fumarase leads to unlabeled fumarate. Similarly, the deuterated L-malate obtained from the enzymatically prepared monodeutero-L-aspartate by chemical conversion with nitrous acid, or by enzymatic conversion in the presence of α-ketoglutarate, transaminase, DPNH, and malic dehydrogenase, yielded essentially unlabeled fumarate when subjected to the action of crystalline mammalian fumarase. These observations are consistent with the idea that the bacterial fumarase and aspartase act similarly to pig heart fumarase and cause deuterium to be added in the same position, in the formation of the methylene group of L-malate and L-aspartate, respectively. The mechanism of the stereospecific enzymatic hydration of fumarate to L-malate by crystalline pig heart fumarase was studied by Farrar et al. (4), who concluded that the enzyme catalyzes a cis addition to the double bond of fumarate. The products of the bacterial fumarase (L-malate) and aspartase (L-aspartate) have the same spatial configuration with respect to the asymmetric carbon atom as the product of the mammalian fumarase reaction. In addition, as shown in the present study, all three enzymes catalyze a deuterium addition specifically to one and the same position on the methylene group of L-malate or L-aspartate. With the use of the monodeuterated product of the mammalian fumarase reaction as a reference model compound, it may therefore be concluded that both the bacterial fumarase and aspartase catalyze a stereospecific cis addition across the carbon-carbon double bond of fumarate.

Concerning the question of a cis versus trans addition to carbon-carbon unsaturations, it may be noted that all four hydrogen positions of succinate are labeled as a result of the anaerobic exchange reaction catalyzed by succinic dehydrogenase preparations (3, 4). In the dehydrogenation of succinate (assuming a staggered configuration), a trans elimination of 2 hydrogen atoms cannot occur with selective action on any one pair of hydrogens; a cis elimination, however, offers a theoretical possibility of a stereochmical distinction between the two pairs of hydrogen atoms. Since all other enzymes studied to date have been shown to behave stereospecifically with respect to methylene hydrogens, it appears likely that succinic dehydrogenase, unlike fumarase and aspartase, catalyzes a trans elimination so that randomly labeled succinate results from the anaerobic exchange reaction in deuterium oxide.

**SUMMARY**

1. *Bacterium cadaveris* aspartase catalyzes a stereospecific addition across the double bond of fumarate. Similar considerations apply to the bacterial fumarase reaction.

2. The deuterated products of the aspartase and bacterial fumarase reaction have the same configuration, with respect to the methylene carbon group of L-aspartate and L-malate, respectively, as the monodeutero-L-malate obtained by the action of crystalline pig heart fumarase in D₂O.

3. With the deuterated product of the mammalian fumarase reaction as a model reference compound, it is concluded that the *Bacterium cadaveris* aspartase and fumarase also catalyze a stereospecific cis addition to the double bond of fumarate.

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