PREPARATION AND PARTIAL PURIFICATION OF THE
 ASPARTASE OF BACTERIUM CADAVERIS*

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Although bacterial aspartase was first demonstrated by Harden (1) in
1901 and later studied in more detail by Quastel and Woolf (2), the first
extensive investigations were those of Gale in 1938 (3). As a consequence
of his experiments with whole cells and cell-free extracts of Escherichia
coli, he proposed that two aspartases exist, one occurring in the albumin
fraction and one in the globulin fraction of cell extracts. These enzymes
were differentiated by their response to adenosine or inosine and their
sensitivity to toluene. The existence of two aspartases has not been con-
firmed by other workers as yet, and there are no additional supporting
data. The most recent studies on bacterial aspartase are those of Lich-
stein and coworkers ((4–6), to cite a few selected references) using Bac-
terium cadaveris, Ellfolk (7–10) using Propionibacterium petersonii and
Pseudomonas fluorescens, and Trudinger (11–13) using Proteus X19. All
of these preparations contained fumarase in addition to other unidentified
enzymes.

The need for continued efforts to obtain an aspartase of higher purity
is apparent, since, until now, any measurement of enzyme characteristics
must be interpreted in the light of influence of accompanying enzymes.
More information is needed on the question of the possible existence of two
aspartases. With these goals in view, we have undertaken the prepara-
tion and purification of the aspartase of B. cadaveris (Gale), since this
organism is highly active in the deamination of aspartic acid.

Methods

Preparation of Cell-Free Extracts—B. cadaveris (Gale) was grown for 16
hours at 30° on a medium consisting of 1 per cent yeast extract, 1 per cent
tryptone, and 0.5 per cent KH₂PO₄. The cells were harvested by centriifu-
gation and washed once with distilled water. From this point every pre-
ceuation was taken to keep the preparation cold and to protect it from
oxidation. The washed cells were then suspended in a small volume of
cold 0.1 m phosphate buffer, pH 6.8, which had previously been boiled

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Francisco, April, 1955.
to expel air. The suspension was placed in the cup of a 9 kc. Raytheon sonic oscillator continuously chilled with circulating ice water, the air above the suspension was displaced by hydrogen, and the cells were then treated for 25 minutes at maximal frequency. The cell débris was removed by centrifugation at 25,000 × g for 30 minutes at 4°. These solutions often were brownish in color because of the presence of colloidal material which was not easily removed. To obtain reasonably colorless solutions for the electrophoresis experiments, it was necessary to increase the centrifugation time to 1 hour. This colloidal material, brown to transmitted light and bluish to reflected light, would eventually spin down as a blue-black sticky paste. After being washed and precipitated a second time, it was found to possess a low degree of aspartase activity, though considerable succinic dehydrogenase activity. For these reasons it was discarded.

Salt Fractionation of Cell Extracts—The clear, straw-colored supernatant solutions obtained as described above were maintained at low temperature and diluted with an equal volume of cold saturated ammonium sulfate solution. The precipitated protein was collected by centrifugation in the cold, redissolved in distilled water, and dialyzed against 0.001 M phosphate buffer, pH 6.8, at 4°. A 24 hour dialysis was usually sufficient to reduce the ammonia content to a level suitable for enzyme assay. The albumin fraction was precipitated by saturation of the remaining supernatant material with solid ammonium sulfate. It was collected, redissolved in distilled water, and dialyzed in the same manner. In some cases salt fractionation was carried out entirely with solid ammonium sulfate.

Electrophoresis Experiments—The globulin fractions obtained by salt fractionation were dialyzed 48 hours at 4° against phosphate buffer of the desired pH and ionic strength. To maintain high activity of the aspartase a small amount of sodium formate was added to the buffer. The precise rôle of formate in the aspartase system is as yet undefined, but its presence greatly enhanced the stability of the preparations studied in our laboratory. Frozen cell extracts which showed gradual loss of activity when retested over a period of 2 to 3 weeks could be kept successfully for months when a small amount of formate was added to the preparation. The electrophoresis experiments were performed in a Klett-Tiselius electrophoresis apparatus.

Determination of Enzyme Activity—The deamination experiments were performed in air at 37° in a variety of buffers of desired pH. For routine testing, 0.05 M phosphate, pH 6.8, and an incubation period of 30 minutes were employed. The reaction was stopped by the addition of 0.1 volume of 25 per cent trichloroacetic acid to the tubes. The precipitated protein was centrifuged, and the supernatant solution analyzed for ammonia by nesslerization and colorimetry in an Evelyn photoelectric colorimeter equipped with a 470 to 490 mμ filter. Protein was determined by the
method of Exton (14), fumarase by the method of Massey (15), and succinic dehydrogenase by the method of Repaske (16).

EXPERIMENTAL

Preliminary Experiments—In the initial phases of the work, many small preparations were made by harvesting the cells from 15 liters of medium and proceeding as described above. The object in view was to study the stability of the aspartase, the extent to which it might be aged and reactivated by various stimulators, and the effect of certain inhibitors. In the first four or five preparations, which were frozen after dialysis against phosphate buffer, there was a gradual decline in activity over a period of 2 or 3 weeks. During this time there was often a significant response to formate and sometimes a small response to adenosine. Repeated experiments with the same preparation made it apparent that these effects were not consistently reproducible, for no obvious reason. A set of data from one of the more successful of these experiments is presented in Table I.

The response to formate was tentatively interpreted as a reactivation of the enzyme by reducing action, since it has been shown by Ellfolk that aspartase possesses sulfhydryl groups, the oxidation of which inactivates the enzyme. The slight stimulation occasionally obtainable with adenosine was observed with both the globulin and albumin fractions (in conflict with the data of Gale) and usually in experiments in which long incubation times were employed. Old preparations with decreased activity showed no significant response to glucose, hydrogen, coenzyme I, leucovorin, ascobic acid, or low concentrations of glutathione, under the

<table>
<thead>
<tr>
<th>Additions</th>
<th>NH₃ per ml.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>γ</td>
</tr>
<tr>
<td>Adenosine, 0.0005 M</td>
<td>3.36</td>
</tr>
<tr>
<td>Sodium formate, 0.005 M</td>
<td>4.28</td>
</tr>
<tr>
<td>Biotin, 0.05 γ per ml.</td>
<td>8.48</td>
</tr>
<tr>
<td>Adenosine + formate</td>
<td>3.86</td>
</tr>
<tr>
<td>&quot; formate, biotin</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>11.1</td>
</tr>
</tbody>
</table>

* The globulin fraction had been stored for 2 weeks. Incubation was carried out at 37° in 0.05 M borate buffer, pH 7.0, for 30 minutes. The concentration of aspartic acid was 0.001 M. All values corrected for ammonia in blanks. 0.67 mg. of protein per ml.
conditions of testing. One preparation, fresh from the dialysis bath, was accidentally left in the laboratory for a period of 20 hours at a temperature of 30°. The unopened dialysis bag lay in a dry beaker, exposed to the air of the laboratory. No putrefaction occurred and the dialysis membrane effectively excluded air from the preparation. Testing of this material showed it to be fully as active as any other of the cell extracts similarly prepared. This observation was taken as an indication that the enzyme may be surprisingly stable to moderate temperatures, though extremely sensitive to oxidation. To avoid the addition of preservatives, however, preparations were stored at -18° and thawed out as needed. These preparations (also containing fumarase and succinic dehydrogenase) showed decreased deamination in the presence of 0.05 M succinate, 0.5 M KI, 0.1 M malonate, and 0.003 M pyrophosphate. No deleterious effects due to the presence of the toluene were observed when incubations were performed under toluene. Attempts to fractionate the crude preparations with salts other than ammonium sulfate and with alcohol were unsuccessful. The deamination was specific for L-aspartic acid.

Purification of Pooled Fractions—To obtain sufficient quantities of cell

### Table II

<table>
<thead>
<tr>
<th>Per cent saturation</th>
<th>Protein (mg. per ml.)</th>
<th>Enzymatic Activity</th>
<th>Optical density (3000 A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-25</td>
<td>10.50</td>
<td>Aspartase 26.6</td>
<td>Fumarase 0</td>
</tr>
<tr>
<td>25-35</td>
<td>13.45</td>
<td>Adenosine 37.7</td>
<td>Succinic dehydrogenase 0.645</td>
</tr>
<tr>
<td>35-45</td>
<td>11.55</td>
<td></td>
<td>0.042</td>
</tr>
<tr>
<td>45-55</td>
<td>5.83</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Above 55</td>
<td>Negative</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

*Incubations carried out at 37° in 0.05 M phosphate buffer, pH 6.8, for 30 minutes. Aspartic acid and adenosine concentrations, 0.002 M. All values corrected for ammonia in blanks.

†Incubations carried out at 25° in 0.033 M phosphate buffer, pH 7.2. Sodium fumarate concentration, 0.0167 M. Protein concentration, 1.35 mg. per ml. at final dilution for 25 to 35 per cent fraction.

‡Incubations carried out at 25° in 0.01 M histidine buffer, pH 6.8. Potassium succinate concentration, 0.009 M. Protein concentration, 0.135 mg. per ml. at final dilution for 25 to 35 per cent fraction; 0.105 mg. per ml. at final dilution for 0 to 25 per cent fraction.
extract for separation in the large cell of the electrophoresis apparatus, eight 15 liter batches of media were harvested over a 2 week period, and the crude globulin fractions were stored at \(-18^\circ\). Since a more exact ammonium sulfate fractionation was desired, a small batch of crude globulin was carefully fractionated so as to give the following separations: 0 to 25, 25 to 35, 35 to 45, and 45 to 55 per cent saturation. These fractions were analyzed for aspartase, fumarase, succinic dehydrogenase, adenosine deaminase, and protein. The results are reported in Table II. The aspartase and adenosine deaminase activities are given on the basis of protein content; the fumarase and succinic dehydrogenase data are not. This distribution of aspartase is not in agreement with the data of Ellfolk, who found most of the activity in the 6 to 25 per cent fraction, but the procedure employed by the latter was quite different from that used in our laboratory. In the case of Ellfolk the higher fractions were allowed to stand for 12 to 18 hours in salt solution and then were dialyzed against tap water (temperature not specified). Since he reports inferior activity in all his ammonium sulfate fractions, it is quite likely that there was much loss of activity because of the conditions used, whereas with our procedure the activity held up well.

On the basis of these findings (particularly the high activity of aspartase in the middle fractions) the combined preparations were fractionated between 25 and 45 per cent saturation. Most of the succinic dehydrogenase was thus eliminated and the fumarase activity was of low order.

**Kinetic Studies**—Since other workers (6, 12) have reported values for the Michaelis constant of the aspartase system, we made similar determina-
### Table III

**Comparison of Various Recent Studies on Aspartase**

<table>
<thead>
<tr>
<th>Author</th>
<th>Organism</th>
<th>Whole cells</th>
<th>Cell-free preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Optimal pH</td>
<td>$Q_{NH_3}$</td>
</tr>
<tr>
<td>Gale (3)</td>
<td><em>E. coli</em></td>
<td>7.5</td>
<td>600</td>
</tr>
<tr>
<td>Trudinger (11, 12)</td>
<td><em>Proteus X19</em></td>
<td>6.8-7.2</td>
<td>&quot;</td>
</tr>
<tr>
<td>Ellfolk (7, 8)</td>
<td><em>P. fluorescens</em></td>
<td>7.5</td>
<td>&quot;</td>
</tr>
<tr>
<td></td>
<td><em>P. peterssonii</em></td>
<td>6.8-7.2</td>
<td>&quot;</td>
</tr>
<tr>
<td>Smith and Lichstein (6)</td>
<td><em>R. cadaveris</em></td>
<td>6.8-7.2</td>
<td>&quot;</td>
</tr>
<tr>
<td>Williams and McIntyre</td>
<td></td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

* Estimated for the purpose of comparison.
† G. = globulin; A. = albumin.
tions for the purpose of comparison. The constant was determined for (a) fresh resting cells, (b) refractionated globulin fraction, and (c) the pooled albumin fractions (not refractionated). These determinations were made at previously determined incubation intervals, selected to give the constant initial rate. A double reciprocal plot for purified globulin is shown in Fig. 1, and the data for all three preparations are presented in Table III. There is a reasonably good agreement among the three experiments and likewise satisfactory agreement with the work of others, which will be discussed later.

Effect of pH—The pH-activity curves for these preparations were also determined for the sake of comparing the albumin and globulin fractions with each other and for comparison with the findings of other workers (Fig. 2). The values for the albumin and globulin fractions were almost identical, as may be seen from Fig. 2, and show a pH maximum around 7.2. In 0.1 M phosphate buffer (data not shown) the maximum was shifted to 6.5 to 6.8.

Electrophoretic Separation Experiment—The electrophoretic separation was carried out in the preparative cell (150 ml.) with phosphate-formate buffer, pH 6.5, ionic strength 0.10, temperature 2.5°, current of 17.5 ma., for 50 hours. Two fractions were then taken from each compartment and
analyzed for aspartase, adenosine deaminase, fumarase, succinic dehydrogenase, and protein. In the compartment containing the most rapidly moving component, a fraction was obtained which was essentially devoid of fumarase, succinic dehydrogenase, and adenosine deaminase, but which possessed the highest activity of aspartase of any of the fractions. Several experiments were performed with this preparation, including the determination of a characteristic rate curve and apparent equilibrium constant for the reaction

$$-\text{OOC-CH$_2$-CHNH$_2$-COO}^- \rightleftharpoons -\text{OOC-CH=CH-COO}^- + \text{NH}_4^+$$

The absence of fumarase and succinic dehydrogenase was regarded as essential to the determination of the true equilibrium constant. The rate

**Table IV**

**Calculation of Apparent Equilibrium Constant for System, Aspartate $\rightleftharpoons$ Fumarate + NH$_3$ (37°C)**

$$K_{eq} = \frac{(-\text{OOC-CH=CH-COO}^-)(\text{NH}_4^+)}{(-\text{OOC-CHNH$_2$-CH$_2$-COO}^-)}$$

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Concentration, moles per l.</th>
<th>Equilibrium constant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>Forward</td>
<td>Aspartic acid 0.0050</td>
<td>0.0050 - 0.0041 = 0.0009</td>
</tr>
<tr>
<td></td>
<td>Fumaric acid 0</td>
<td>0.0041 (from NH$_3$ data)</td>
</tr>
<tr>
<td></td>
<td>Ammonia 0</td>
<td>0.0041 (by analysis)</td>
</tr>
<tr>
<td>Reverse</td>
<td>Fumaric acid 0.0050</td>
<td>0.0050 - 0.0009 = 0.0041</td>
</tr>
<tr>
<td></td>
<td>Ammonia 0.0069</td>
<td>0.0060 (by analysis)</td>
</tr>
<tr>
<td></td>
<td>Aspartic acid 0</td>
<td>0.0009 (from ammonia data)</td>
</tr>
</tbody>
</table>

curve obtained is shown in Fig. 3, and the calculation of the equilibrium constant in 0.05 M phosphate, pH 6.8, for both the forward and the reverse reactions is given in Table IV. Fair agreement was obtained in the determinations.

**DISCUSSION**

Since this study deals principally with the preparation of an aspartase of higher purity and activity than hitherto reported and with the determination of certain characteristics of this enzyme, no attempt will be made to discuss the postulated coenzymes, stimulators, or inhibitors of the system, or the confused state of the literature on this subject. This matter will be discussed in a later publication. In our experience with cell-free preparations, formate was the only addition which increased the activity of the enzyme in a consistent way. At present, this is interpreted to mean a reactivation through reduction of essential sulfhydryl groups.
The Lineweaver-Burk treatment was applied to the velocity data obtained over the substrate concentration range 0.01 to 0.10 M with washed cells, refractionated globulin preparation, and albumin preparation. The double reciprocal plot (not shown) for washed fresh cells was satisfactorily linear throughout and yielded a $K_m$ value of $8 \times 10^{-3}$ as compared with $5 \times 10^{-3}$ obtained by Smith and Lichstein and $8 \times 10^{-3}$ reported by Trudinger (see Table III). Smith and Lichstein report that linearity could not be achieved with the Lineweaver-Burk treatment of velocity-concentration data obtained with washed cells suspended in buffer and substrate alone. With the addition of glucose to the mixture, satisfactory data were obtainable. No such addition was found necessary in the case of our cell preparations. Since the cells studied by Smith and Lichstein were grown on a different medium from that employed in this laboratory, there might possibly be a difference in cell permeability. The effect of glucose on the stimulation of the deamination of aspartic acid by whole cells is at present interpreted to be a permeability effect (6). Fig. 1, a Lineweaver-Burk plot for refractionated globulin, shows a departure from linearity at the higher concentration levels. This effect was consistently observed in a number of experiments and is at present interpreted to mean that the substrate at high concentrations has an activating effect on the free enzyme when there is no membrane to control the rate at which the substrate may reach the enzyme.

In Table III a comparison is made of some of the sources and characteristics of the enzyme as reported by various laboratories. Unfortunately, we have no kinetic data from Ellfolk, and it cannot be safely estimated from the published reports, since in most cases the first time interval was at least 4 hours. The initial rate is not sustained for nearly this length of time. Such data yield a very low $Q_{NH_3}$ value of the order of 10 to 50. In cases in which comparisons can be made, good agreement is obtained by Trudinger, Lichstein, and this report on the determination of $K_m$, despite differences in choice of organism and experimental conditions. In the case of the optimal pH of the enzyme, there is even more unanimity. The values of $Q_{NH_3}$ are given principally to show that the activity as determined in our laboratory is actually much higher than any hitherto reported, presumably because of the increased purity of the preparation. An examination of the data for the globulin and albumin fractions favors the case for one aspartase, since the pH optimum and the Michaelis constant are almost identical. The fact that the albumin fraction contains aspartase activity appears to be attributable simply to the fact that the enzyme as prepared by us and by Gale (3) is not completely insoluble in 50 per cent ammonium sulfate, as may be seen from inspection of Table II.

Adenosine deaminase activity was determined because of interest in the

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2 Micrograms of ammonia per mg. of protein per hour.
hypothesis proposed (and rejected) by Gale that the equilibrium

\[
\text{Inosine} + \text{ammonia} \rightleftharpoons \text{adenosine}
\]

might be linked to the aspartase-catalyzed deamination and thus account for the stimulating effect of inosine and adenosine on this system. The hypothesis was rejected by Gale on the basis of incompatibility of the reaction rates. Our evidence corroborates the viewpoint that the two enzymes have no direct relationship, since the electrophoretically purified aspartase fraction contained insignificant amounts of adenosine deaminase, but proved to have the highest aspartase activity as yet measured in our laboratory.

The values obtained for the equilibrium constant under the conditions of temperature and pH employed in these experiments agreed with our innumerable observations that the equilibrium lies well to the right. It is likewise our observation that in the presence of fumarase and other related enzymes the deamination may often go to completion. In the absence of such outlets for fumarate, however, the balance point appears to lie at about 80 per cent deamination of aspartate.

SUMMARY

An aspartase of superior purity and activity has been prepared from cell-free extracts of \textit{Bacterium cadaveris} (Gale).

Cell extracts purified by salt fractionation and electrophoresis exhibited activities of the order of 6000 and a pH optimum of 7.2. The Michaelis constant for the purified cell extracts was found to be of the order of \(2 \times 10^{-2}\); for resting cells the constant was \(8 \times 10^{-3}\).

The most highly purified fraction, containing insignificant amounts of fumarase and succinic dehydrogenase, was used to determine the apparent equilibrium constant for the reaction

\[
-OOC\cdot\text{CII}_2\cdot\text{CHNH}_3^+\cdot\text{COO}^- \rightleftharpoons -OOC\cdot\text{CII}^-\cdot\text{CH}^+\cdot\text{COO}^- + \text{NH}_4^+
\]

At \(37^\circ\), pH 6.8, in 0.05 M phosphate, \(K'_{\text{eq.}} = 2 \times 10^{-2}\).

The small amount of aspartase which precipitated with the albumin fraction was found to possess the same characteristics as the larger quantity of the enzyme found in the globulin fraction, and hence was assumed to be the same enzyme.

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BIBLIOGRAPHY

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