THE RÔLE OF BIOTIN AND ADENYLIC ACID IN AMINO ACID DEAMINASES

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(Received for publication, May 3, 1948)

Within the past year the mode of action of biotin has become increasingly clarified. The relation of this vitamin to oxalacetic acid decarboxylation was discovered independently in at least four laboratories (1-4), while its apparent rôle in the synthesis of oleic acid has been suggested by Williams et al. (5) and Snell et al. (6). Biotin has further been shown to activate the deamination of aspartic acid, serine, and threonine (7). This paper is concerned with biotin activation of the deaminases.

Methods

The organisms employed in these studies were Escherichia coli (Gratia), Escherichia coli (10B3), Proteus vulgaris, Aerobacter aerogenes, and Bac- terium calaveris (Gale). They were grown for approximately 16 hours at 27-30° in a medium composed of 1 per cent each of tryptone and yeast extract and 0.5 per cent K$_2$HPO$_4$; the final pH was 6.9 to 7.2. In certain instances this medium was supplemented with 0.1 per cent formate. Cells were harvested by centrifugation, washed once with distilled water, and suspended in M phosphate, pH 4, to give about 1 mg. of bacterial nitrogen per ml. Biotin deficiency was obtained as previously described (3, 7) by holding the cell suspensions at pH 4 in phosphate buffer for 30 to 60 minutes at 20-30°. The deamination experiments were performed at either pH 4 or 7 in phosphate buffer at 37°. After incubation in the presence of an amino acid substrate the reaction was stopped with 100 per cent trichloroacetic acid, and ammonia was determined colorimetrically (Klett-Summerson photoelectric colorimeter) on aliquots of the centrifuged samples. The biotin employed was the free form$^1$ and the adenylic acid was the adenosine-5-phosphoric acid.$^2$ Further details are included with the data.

Results

The data given in Table I demonstrate that biotin stimulates aspartic, serine, and threonine deaminases. Biotin alone fully replaces the mixture

$^1$ We are grateful to Merck and Company, Inc., Rahway, New Jersey, for supplies of this material.

$^2$ Kindly supplied to us by the Ernst Bischoff Company, Ivoryton, Connecticut.
of all the known members of the vitamin B complex, while such a mixture without biotin gives little or no stimulation.

**Table I**

*Biotin Activation of Aspartic Acid, Serine, and Threonine Deaminase*

Cells grown as described in the text, aged at pH 4 in m phosphate at 25-30°. Reaction run at pH 7, 37°, 20 to 30 minutes; volume 2 ml. Amino acids added at 0.005 m final concentration. The increase in ammonia over samples stopped at zero time was taken as an index of deamination.

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* Vitamins added per ml., nicotinic acid 2.5 γ, p-aminobenzoic acid 1 γ, riboflavin 0.5 γ, pantothenic acid 0.5 γ, thiamine 1 γ, folic acid 0.5 γ, pyridoxal 5 γ.
† As vitamins less biotin with free biotin added to yield 0.1 γ per ml. of the reaction mixture.

Attempts to reverse deamination of serine and threonine have so far been unsuccessful, while with aspartic acid such reversal is readily demonstrable. Using NH₄Cl and malic acid, we have been able to follow the
disappearance of ammonia and to show that biotin and adenylic acid are also involved in this reaction. Representative data are given in Fig. 1. Results with fumarate have been variable, probably because of permeability effects. We have previously shown (3) that the product of aspartic acid deamination in these cells is fumaric acid and not malate, but that malate and fumarate exist in equilibrium because of an active fumarase present in these cells.

Biotin fails to stimulate the deamination of alanine, phenylalanine, and methionine (chosen as typical of the substrates of the D- or L-amino acid oxidases) and glutamic acid in comparable experiments in which it was effective in stimulating aspartic deaminase (Table II). The glutamic deaminase in Aerobacter aerogenes has given variable results, as shown by the data in Table II. In one of five experiments performed, a definite stimulation with biotin was recorded. However, to determine whether this is a direct biotin effect on glutamic deaminase or an indirect action due to transamination of glutamic acid to aspartic acid (with oxalacetic acid present in the cell) and subsequent deamination of the latter amino acid requires further study. It may be noted that glutamate deamination is a

Fig. 1. Reversibility of aspartic acid deaminase in Bacterium cadaveris. Cells aged at pH 4 in m phosphate at 20° for 60 minutes. Reaction run at pH 7, 37°. 0.03 m malic acid + 20 γ of ammonia as NH₄Cl. Reaction volume 2 ml.
somewhat different process than aspartate deamination in that the former is reported to involve a diphosphopyridine nucleotide- or a triphosphopyridine nucleotide-linked dehydrogenation with the formation of the keto acid (8).

Gale (9, 10), working with aspartic and serine deaminases in *Escherichia coli*, reported that washed suspensions of these cells lost activity on standing and that the activity could be recovered by adding boiled cell suspensions or other materials. Further work showed that the killed cell substance could be replaced by adenylic acid and its breakdown products, the most active of which was adenosine. However, this was only true for aspartic deaminase, since adenylic acid in very low concentrations prevented both loss of activity and recovery in the case of serine deaminase. It was there-

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fore of interest to determine whether adenylic acid stimulated the deficient cells obtained by our aging technique, which respond to biotin. The results of such experiments (Table III) show that muscle adenylic acid can replace biotin in aspartic, serine, and threonine deaminase, and that no additive effect is obtained by combining adenylic acid and biotin. Although the data presented here with aspartic deaminase are in general agreement with those of Gale (9), our results with serine deaminases are distinctly at variance with Gale's work (10).

**Table III**

**Adenylic Acid Stimulation of Aspartic Acid, Serine, and Threonine Deaminase**

Conditions as for Table I.

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Further experiments were designed to determine the relative levels of each substance required to stimulate aspartic deaminase in *Bacterium cadaveris*. The data (Table IV) show a striking difference in the concentration of biotin and of adenylic acid required to activate this enzyme. In Experiment 1 the aging process resulted in cells which were completely unable to deaminate aspartic acid without additions. The biotin levels tested were 0.001 to 5 \(\gamma\) per 2 ml. of reaction volume. All levels were effective and no end-point was reached. On the other hand, 1 \(\gamma\) of adenylic acid seemed to be necessary for good stimulation and 0.1 \(\gamma\) gave very little activity. In Experiment 2 the dilutions of both substances were carried
further in an attempt to get a good end-point. The aging process was not as effective as in Experiment 1, but significant stimulations were obtained.

**Table IV**

*Relative Concentration of Adenylate Acid and Biotin Required to Stimulate Aspartic Deaminase in Bacterium cadaveris*

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<th>Experiment No.</th>
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The data obtained show that $10^{-5}$ γ of biotin is sufficient to stimulate the aged cells fully, whereas the activity of $10^{-6}$ γ is considerably less. The
end-point for adenyllic acid is somewhere between 1 and 10 \(\gamma\); so that biotin is roughly 100,000 times more effective than adenyllic acid. In Experiment 3 the aging process was very effective, resulting in cells showing very limited activity. Significant stimulations were recorded for biotin through \(10^{-5}\) \(\gamma\) per 2 ml., while 0.1 \(\gamma\) of adenyllic acid was required for similar stimulation. In Experiment 3 biotin was about 10,000 times more effective than adenyllic acid. The deficiency obtained in Experiment 4 was almost identical with that in Experiment 2. Significant biotin stimulations were obtained through \(10^{-4}\) \(\gamma\) per 2 ml., while adenyllic acid was required in a concentration of 0.1 \(\gamma\) per 2 ml. to give equal stimulation, again making biotin about 10,000 times more effective than adenyllic acid (see Fig. 2).

![Graph showing relative concentrations of adenyllic acid and biotin required to stimulate aspartic acid deaminase in Bacterium cadaveris.](http://www.jbc.org/)

The logical question arises as to the rôle of adenyllic acid in the deamination of aspartic acid, serine, and threonine. Although on the basis of the data cited (Table IV) adenyllic acid is roughly 10,000 times less effective in stimulating the deficient cells than is biotin, it does nevertheless fully stimulate the aged cell. Controlled experiments show that under the conditions of the experiments there is no ammonia released from adenyllic acid itself; so that increases in ammonia are due to deamination of added amino acid.

Several hypotheses were considered in an attempt to clarify the rôle of biotin and adenyllic acid in the activation of these amino acid deaminases. The first of these was that the adenyllic acid employed in these studies contained biotin as a contaminant. Such a hypothesis could be tested experimentally by microbiological assay for biotin. The methods em-
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ployed were those given by Snell et al. (11) with Saccharomyces cerevisiae. The results obtained from such assays revealed that 1 γ of adenylic acid was equivalent to $10^{-10} \gamma$ of biotin on the basis of stimulation of the growth of Saccharomyces cerevisiae in a chemically defined medium lacking only in biotin. Since to account for the activation of the deaminases the adenylic acid should have contained $10^{-4} \gamma$ of biotin per microgram, it is unlikely that adenylic acid stimulation of aspartic, serine, and threonine deaminases is due to its biotin content.

Hypotheses other than biotin contamination of adenylic acid were developed; namely, (1) biotin may not be the coenzyme of these deaminases but functions in some manner in the production of adenylic acid, (2) adenylic acid may function as a non-specific energy source supplying the energy necessary to synthesize the active coenzyme form of biotin, (3) adenylic acid may be specifically necessary to phosphorylate biotin, (4) adenylic acid may combine with biotin to form a coenzyme similar in structure to diphosphopyridine or triphosphopyridine nucleotide. Attempts were made to put these hypotheses to experimental test.

It was felt that time curves would shed light on the first hypothesis, since, if biotin activation of these deaminases is due to an indirect effect, namely its necessity in the formation of adenylic acid, then there should be a definite lag period before biotin stimulation is noted and essentially none with adenylic acid. The data (Table V) presented are for six typical experiments. In all cases biotin stimulation was noted before adenylic acid stimulation, and in all but one instance biotin activation was present at the first time interval. These data suggest that the first hypothesis is unsound and that biotin must be directly associated with the aspartic acid deaminase. It must be pointed out that the lag in adenylic acid stimulation could well be due to permeability factors in the living cell. It should be noted that in three of the six experiments cited, in which the time curves were extended to 60 minutes, adenylic acid stimulation of aspartate deaminase at pH 7 exceeded that produced by biotin. This was taken as an indication that adenylic acid may be serving as an energy source either specifically or non-specifically (hypotheses (2) and (3)) and that the aged cells lose the ability to synthesize the coenzyme form of biotin in the absence of a suitable energy supply. Similar results have been obtained for serine and threonine deaminases. Further experiments were designed to study biotin and adenylic acid stimulation at pH 7 and 4 since the latter pH should be low enough to reduce markedly or even inhibit entirely the synthetic mechanisms of the cell. It was also considered advisable to include the breakdown products of adenylic acid, namely adenine and ribose, and to check the specificity by using guanine and xy-
lose. Results of three typical experiments are given in Table VI and the data of one are graphically presented in Fig. 3.

It may be seen that the activation of aspartic acid deaminase by all substances tested with the exception of biotin differs at pH 7 and 4. Stimulation by adenine, guanine, xylose, and ribose, while noted at pH 7, is not present at pH 4, suggesting that these substances may stimulate only under conditions which enable the cells to gain energy from them to synthesize the coenzyme. That synthesis of the aspartate deaminase coenzyme does occur even in the absence of added substances may be seen from a comparison of the figures for no additions at pH 7 and 4. Such a comparison shows that at pH 7 the cells can synthesize the coenzyme, while at pH 4 this synthesis is either absent or greatly reduced. Biotin stimulation of aspartate deaminase seems not to depend on the pH, although it is usually more pronounced at pH 4 than at pH 7 because of the inability of the cells to synthesize the coenzyme without this vitamin at the former pH. Stim-

**Table V**

*Time Required for Biotin and Adenylic Acid Stimulation of Aspartic Acid Deamination in Bacterium cadaveris*

Conditions as for Table I.

The figures represent the micrograms of ammonia nitrogen produced after subtracting the micrograms of ammonia present in similar cell suspensions incubated at the times given without added aspartate.

<table>
<thead>
<tr>
<th>Additions</th>
<th>0 min</th>
<th>1 min</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
<th>20 min</th>
<th>30 min</th>
<th>60 min</th>
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<tbody>
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<td>4.2</td>
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<td></td>
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<td></td>
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<td>4.6</td>
<td>7.3</td>
<td>9.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>3.7</td>
<td>6.4</td>
<td></td>
<td></td>
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<td>7.7</td>
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<td>9.5</td>
<td></td>
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<tr>
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<td>1.1</td>
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<td>6.9</td>
<td>7.6</td>
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<td>0</td>
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<td>4.0</td>
<td>6.6</td>
<td>20.2</td>
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<td>4.0</td>
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<td>6.6</td>
<td>20.2</td>
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<td></td>
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<td>4.6</td>
<td>6.8</td>
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<td>10.0</td>
<td>25.6</td>
<td>29.6</td>
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<td>33.0</td>
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<td>10.5</td>
<td>10.0</td>
<td>16.0</td>
<td>33.0</td>
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<td></td>
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<td>33.0</td>
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<td></td>
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<tr>
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<td>4.6</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>0.5 γ biotin</td>
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<td>10.5</td>
<td>10.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>11.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</table>
TABLE VI
Effect of Various Substances on Deamination of Aspartic Acid by Bacterium cadaveris

<table>
<thead>
<tr>
<th>Additions per ml.</th>
<th>Ammonia nitrogen produced</th>
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<tr>
<td></td>
<td>pH 7</td>
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<tr>
<td></td>
<td>30 min.</td>
</tr>
<tr>
<td>None</td>
<td>4.6</td>
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<tr>
<td>0.05 γ biotin</td>
<td>9.1</td>
</tr>
<tr>
<td>50 γ adenylic acid</td>
<td>8.9</td>
</tr>
<tr>
<td>50 &quot; adenine</td>
<td>5.0</td>
</tr>
<tr>
<td>50 &quot; guanine</td>
<td>4.6</td>
</tr>
<tr>
<td>50 &quot; ribose</td>
<td>5.7</td>
</tr>
<tr>
<td>50 &quot; xylose</td>
<td>5.2</td>
</tr>
</tbody>
</table>

|                  | 20 min. | 40 min. | 60 min. | 30 min. | 40 min. | 80 min. |
| None             | 13.5   | 15.3   | 22.2   | 2.6     | 2.6     | 3.4     |
| 0.05 γ biotin    | 15.5   | 17.4   | 21.8   | 4.0     | 6.5     | 11.4    |
| 50 γ adenylic acid | 15.5  | 21.0   | 23.9   | 2.8     | 2.3     | 9.5     |
| 0 05 γ biotin + 50 γ adenylic acid | 15.5  | 22.4   | 25.8   | 4.0     | 6.1     | 16.0    |
| None             | 8.3    | 11.6   | 18.9   | 1.4     | 1.8     | 2.2     |
| 0.05 γ biotin    | 12.1   | 16.2   | 25.3   | 6.9     | 10.3    | 14.6    |
| 50 γ adenylic acid | 10.3  | 16.4   | 29.8   | 1.9     | 2.3     | 9.6     |
| 0.05 γ biotin + 50 γ adenylic acid | 12.6  | 20.1   | 33.6   | 7.3     | 10.0    | 17.3    |

Fig. 3. Effect of pH on biotin and adenylic acid stimulation of aspartic acid deamination in Bacterium cadaveris.
ulation by adenylic acid, while differing at pH 7 and 4, is definitely present in both instances as opposed to adenine, guanine, ribose, and xylose. It is noted that, whereas adenylic acid stimulation at pH 7 usually exceeds biotin (Tables V and VI) after a suitable incubation period, at pH 4 the lag period before adenylic acid stimulation is noted is greatly extended and after 80 minutes incubation it has not reached the biotin activity. It is of definite interest to note that in some instances (Table VI and Fig. 3) cells may be stimulated to a greater extent by a combination of biotin and adenylic acid than by either agent alone.

The data of Table VII show that on two occasions cells were obtained which at pH 4 failed to be stimulated by either biotin or adenylic acid alone, but were markedly stimulated by a combination of biotin and adenylic acid. These same cells at pH 7 were able to synthesize coenzyme, biotin activity and adenylic acid stimulation were typical, and very slight if any additive effect is noted when both biotin and adenylic acid are combined. These data suggest that the third or fourth hypothesis may be valid; namely, that adenylic acid is intimately associated with these deaminases, either having the function of phosphorylating biotin to an active coenzyme form or by chemically combining with biotin to form the coenzyme.

It might be well to consider in some detail the aging process as employed in our studies to obtain a biotin deficiency. Microbiological assays for biotin content in fresh and aged suspensions of *Bacterium cadaveris* have shown a definite reduction in biotin content after aging at pH 4 in phosphate

### Table VII

**Effect of Biotin Plus Adenylic Acid on Aspartic Acid Deaminase in Bacterium cadaveris**

<table>
<thead>
<tr>
<th>Additions per ml.</th>
<th>pH 4</th>
<th>pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min.</td>
<td>60 min.</td>
</tr>
<tr>
<td>None</td>
<td>γ</td>
<td>γ</td>
</tr>
<tr>
<td>0.05 γ biotin</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>50 γ adenylic acid</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>0.05 γ biotin + 50 γ adenylic acid</td>
<td>2.8</td>
<td>8.6</td>
</tr>
<tr>
<td>None</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>0.05 γ biotin</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>50 γ adenylic acid</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>0.05 γ biotin + 50 γ adenylic acid</td>
<td>1.9</td>
<td>6.5</td>
</tr>
</tbody>
</table>
EFFECT OF BIOTIN ON DEAMINASES

buffer. Thus in one typical experiment a freshly harvested bacterial suspension contained $10^{-3}$ γ of biotin per mg. of cell nitrogen, while after aging at pH 4 in M phosphate for 30 minutes at 30° it contained $10^{-5}$ γ of biotin per mg. of cell nitrogen, or about a 100-fold reduction.

It must be emphasized, as is already evident from some of the data presented, that the degree of biotin deficiency obtained by the aging process varies greatly. At times, although rarely, cells are obtained which are completely unable to deaminate aspartic acid in the absence of added biotin, while at other times, again rarely, cells may be obtained which are unable to deaminate aspartic acid, even upon the addition of biotin. In general the aging technique when carefully controlled will result in cells that show some deficiency in a reaction involving biotin, and biotin stimulation is recorded.

Although the mechanism of the aging process is not known, we feel that it can occur both enzymatically and non-enzymatically, but in living cells the former is probably the case. The mechanism may be a destruction of an active coenzyme form of biotin at pH 4. The following data are in support of this hypothesis. Free biotin can be added at pH 4 and stimulation of the deficient cells occurs, suggesting that biotin can itself be converted to the coenzyme form at this pH. Biotin solutions may be kept at pH 4 without loss in activity, while yeast extract, which can replace biotin in the stimulation of these enzymes, loses its activating effect after 4 to 12 hours at pH 4 at ice box temperatures. In certain instances yeast extract kept at pH 4 will not only lose its stimulatory effect but may show some inhibition. These findings suggest that yeast extract may contain some of the coenzyme form of biotin which is non-enzymatically degraded to an inactive form and in some cases to an inhibitory analogue. While these data do not prove the existence of another active form of biotin, they are suggestive that a coenzyme form does exist. It may be noted that so far all the vitamins which have been shown to function as coenzymes exist in an active phosphorylated form. As to whether or not the aging or splitting phenomenon is an enzymatic one, two other findings should be mentioned. In our experience cells grown in the absence of yeast extract cannot be made biotin-deficient by our technique, and, secondly, living cells can be aged at pH 4, while vacuum-dried preparations have so far given negative results. Both of these findings are suggestive of an enzymatic aging process, since one may postulate that some factor in yeast extract is required for the activity of the enzyme which cleaves the holoenzyme into the apoenzyme and coenzyme, and that this cleaving enzyme is labile to vacuum drying. Certainly if the aging process was merely a matter of
diffusion of a cofactor from the cell into the suspending menstruum, we would expect the process to occur as readily or more so in a vacuum-dried preparation of the same cells.

We have investigated to some degree the optimum conditions for aging and to date can recommend the following as giving the best results in our hands: growth conditions, tryptone gives better results than peptone; the addition of formate is advantageous; yeast extract is essential; aging conditions, a cell concentration of 1 mg. of N per ml. or less gives best results; phosphate at pH 4 is better than at pH 3 or 5; 20° is better than 10° or 30°.

The data presented in this paper show that both biotin and adenylic acid are concerned with the activation of aspartate, serine, and threonine deaminases. Although the mechanism by which these substances stimulate is not yet entirely clear, the results suggest that biotin exists in a coenzyme form and that adenylic acid is functioning either as a phosphorylating agent of biotin to an active coenzyme or that it combines with biotin to form a coenzyme, possibly similar in structure to diphosphopyridine or triphosphopyridine nucleotide. The studies reported here do not enable one to distinguish between these two or even more possibilities.

It is relevant to add another point regarding biotin and adenylic acid stimulation; namely, whether or not the coenzyme can be assayed. On several occasions microbiological assays for biotin were made on experimental tubes and it was found that biotin could be detected in all instances equal to the amount added plus the amount originally present in the cells. In the case of adenylic acid stimulation no increase in biotin content was found. This may be interpreted as a suggestion that adenylic acid stimulation is an indirect one, having nothing to do with the production of biotin. However, when we consider that in the case of biotin activity no change in biotin content was found, it may be either that the amount of coenzyme formed is so small as not to be detected by our assay methods or that the active biotin coenzyme is not assayed by the method employed.

SUMMARY

Biotin deficiency of several bacterial species was obtained by holding the cells at pH 4 in phosphate buffer at 20-30° for 30 to 60 minutes. Such cells show a markedly decreased ability to deaminate aspartate, serine, and threonine and this activity is restored by the addition of biotin or adenylic acid to washed cell suspensions. Cells similarly treated show no difference in alanine, phenylalanine, methionine, and glutamic acid deaminase activity. Experiments are presented in an attempt to clarify the rôle of biotin and adenylic acid in aspartate, serine, and threonine deaminases.
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THE RÔLE OF BIOTIN AND ADENYLIC ACID IN AMINO ACID DEAMINASES
Herman C. Lichstein and John F. Christman


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