SYNTHESIS AND DETERMINATION OF THE LIPOTROPIC ACTIVITY OF THE BETAINES HYDROCHLORIDES OF Dl-SERINE, Dl-THREONINE, AND Dl-ALLOTHREONINE*

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This paper reports the synthesis of the betaine hydrochlorides of serine, threonine, and allothreonine and a study of the action of these substances on the lipid content of the livers of rats fed a high fat, low protein diet. This work was undertaken for two purposes. Best and Huntsman (1) found that choline prevented the development of fatty livers in rats fed a high fat diet and that betaine possessed a similar lipotropic effect. Since choline contains a hydroxyl group, it was of interest to determine the lipotropic activity of some hydroxybetaines which are closely related to choline in structure.

It also seemed possible that these studies might throw some light on the origin of choline in the animal body. The discovery of Channon and Wilkinson (2) and of Best and Huntsman (3) that casein has a lipotropic effect has been interpreted as indicating the possible synthesis of choline from one of the constituent amino acids of casein. Of the amino acids of casein, serine, containing the hydroxyethylamine grouping, is most closely related structurally to choline. Hence the physiological behavior of serine betaine is of particular interest, although it represents only one of

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† The experimental data in this paper are taken from a thesis submitted by Donald B. Melville in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate School of the University of Illinois.
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the possible intermediates by which serine might be converted into choline in vivo.

In the course of the synthetic work it was discovered that the betaines of threonine and allothreonine (but not of serine) are unstable in alkaline solutions, undergoing a retrograde aldol condensation as shown in the accompanying equation.

\[
\begin{align*}
\text{CH}_3\text{CH} & \quad \text{CH} \quad \text{C} & \quad \text{O} \\
\text{OH} \quad \text{N}^+ \quad \text{(CH}_3)_2 & \quad \text{CH}_2\text{C} & \quad \text{O} \\
\end{align*}
\]

Preliminary experiments indicate that this reaction offers promise as a method for the quantitative determination of threonine. A study of other hydroxybetaines has revealed the fact that this reaction is a general one for \( \alpha \)-amino-\( \beta \)-hydroxy acids and may be useful in differentiating these compounds from the isomeric \( \beta \)-amino-\( \alpha \)-hydroxy acids.

EXPERIMENTAL

Preparation of Betaine Hydrochlorides

The preparation of the betaines was first attempted by the method of Novak (4). 11.9 gm. of \( dl \)-allothreonine, dissolved in 100 cc. of 1.0 \( N \) potassium hydroxide, were methylated with 56.7 gm. (0.45 mole) of dimethyl sulfate and 100 cc. of 4.5 \( N \) potassium hydroxide. The alkaline reaction mixture was then refluxed in order to destroy any unchanged dimethyl sulfate. The solution immediately became hazy and a dark brown precipitate gradually separated. At the end of 30 minutes the mixture was cooled and filtered. The solution had a strong odor of acetaldehyde. The filtrate was decolorized with norit and worked up in the usual manner. The crude product was recrystallized from alcohol, giving 5.0 gm. of a crystalline compound melting at 236\(^\circ\)-238\(^\circ\). The analytical data indicated that this substance was betaine hydrochloride instead of the expected allothreonine betaine hydrochloride.

\[
\begin{align*}
\text{C}_7\text{H}_{14}\text{O}_2\text{NCl} & \quad \text{Calculated. N} & \quad 7.09, \text{ Cl} & \quad 17.97 \\
\text{C}_7\text{H}_{12}\text{O}_2\text{NCl} & \quad \text{"} & \quad 9.11, \text{ "} & \quad 23.13 \\
\text{Found.} & \quad \text{"} & \quad 9.07, \text{ "} & \quad 23.47
\end{align*}
\]
The identity of the substance was confirmed by the preparation of the picrate which melted at 180–182° and gave no depression of the melting point when mixed with an authentic sample of betaine picrate. Evidently allothreonine betaine is unstable in an alkaline medium, undergoing a retrograde aldol condensation with the production of betaine and acetaldehyde. The latter compound polymerizes under the influence of alkali, giving the gummy brown precipitate which appears during the refluxing of the alkaline methylation mixture.

Under the above conditions dl-threonine betaine also decomposes to betaine and acetaldehyde. dl-Serine betaine, however, is more stable toward alkali and no evidence was obtained for the production of formaldehyde when the methylation mixture was heated. In this case the product, isolated in the usual manner, was a hygroscopic syrup which crystallized after long standing. A low yield (10 to 20 per cent) of dl-serine betaine hydrochloride was obtained when this material was recrystallized several times from absolute alcohol.

In an attempt to avoid these difficulties Novak's procedure was modified as follows: The methylation was carried out as before. The cold solution was then acidified with hydrochloric acid (100 cc. of 5.0 N per 0.1 mole of amino acid methylated) and the acid solution was refluxed for 5 hours. In this way both dimethyl sulfate and monomethyl sulfate were hydrolyzed in a single step. The betaine hydrochlorides were isolated as in Novak's procedure. In each case the product was a hygroscopic syrup which crystallized very slowly. Only a small amount of pure betaine hydrochloride could be obtained from the crude products and the purification required repeated recrystallizations. Since an excess of dimethyl sulfate is used in Novak's method, it was possible that a certain amount of O-methylation had occurred. Strong evidence supporting this view was the fact that the neutral equivalents of the partially purified products were 2 to 4 per cent higher than the calculated value. In the case of serine the O-methyl betaine hydrochloride was actually isolated in a pure state. The crude product obtained in one run was dissolved in absolute alcohol and cooled in an ice bath. An oil precipitated immediately. On further standing, large flat crystals appeared on the sides of the flask. These were carefully removed and re-
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crystallized from absolute alcohol. The product thus obtained melted at 196-197°, and gave the correct analytical data for O-methyl-dl-serine betaine hydrochloride.

\[ \text{C}_7\text{H}_{16}\text{O}_3\text{NCl} \]

Calculated. N 7.09, Cl 17.97, neutral equivalent 197.5

Found. N 7.02, Cl 17.93, " " 200.0

This difficulty was overcome by decreasing the amount of dimethyl sulfate and by altering the conditions of the reaction slightly. Satisfactory results were finally obtained by the following procedure.

A solution of 0.1 mole of amino acid in 100 cc. of 1 N potassium hydroxide was placed in a 500 cc. 3-necked flask equipped with a stirrer and two small separatory funnels. 37.8 gm. (28 cc., 0.3 mole) of dimethyl sulfate were placed in one separatory funnel, and a solution of 16.8 gm. (0.3 mole) of potassium hydroxide in 28 cc. of water was placed in the other. The flask was cooled in an ice bath and the two liquids were added at the same rate to the vigorously stirred reaction mixture over a period of 30 minutes. 40 cc. of concentrated hydrochloric acid were added and the solution was refluxed for 5 hours. 75 gm. of barium chloride dihydrate in 200 cc. of water were added to the warm solution. The barium sulfate was removed by filtration and washed twice with warm water. The filtrate and washings were concentrated to a syrup in vacuo. The residue was extracted with 300 cc. of absolute alcohol and the insoluble material (potassium chloride) was removed by filtration. The filtrate was concentrated and the residue was dissolved in 100 cc. of absolute alcohol. The alcohol was removed under reduced pressure, giving the crude betaine hydrochloride in the form of a viscous oil. The purification of the individual products is described below.

dl-Serine Betaine Hydrochloride—The crude product was dissolved in 100 cc. of warm absolute alcohol. The solution was filtered, and cooled in the ice box for 48 hours. The crystalline precipitate was removed by filtration, washed with cold alcohol and ether, and dried in a vacuum desiccator. 9 gm. of dl-serine betaine hydrochloride were thus obtained. A small additional amount (3 gm.) of impure material precipitated from the alcoholic filtrate on the addition of ether. This was purified by recrystal-
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Hydroxylation from 5 volumes of hot absolute alcohol. The total yield of pure product, melting at 198–199°, was 10.8 gm. (58 per cent).

\[ \text{C}_{12} \text{H}_{16} \text{O}_{2} \text{NCl} \]

Calculated. N 7.63, Cl 19.35, neutral equivalent 183.5

Found. N 7.64, Cl 19.10, " 185

\textit{dl-Serine betaine hydrochloride} is slightly hygroscopic and is highly water-soluble. It is moderately soluble in ethyl and butyl alcohol and is best purified from the former solvent.

\textit{dl-Allothreonine Betaine Hydrochloride}—The crude product was placed in a vacuum desiccator over phosphorus pentoxide for 6 days, during which time the substance slowly crystallized. The crystalline mass was dissolved in 50 cc. of hot absolute alcohol. The solution was filtered and cooled to room temperature. Anhydrous ether was added slowly until a faint haziness was produced. The solution was seeded and placed in the ice box overnight. The crystalline precipitate was removed by filtration, washed with ether, and air-dried. This material was recrystallized again from an alcohol-ether mixture, giving 9 gm. (45 per cent yield) of pure \textit{dl-allothreonine betaine hydrochloride} melting at 166–168°. A small additional amount of product may be obtained by working up the filtrates, but it is hardly profitable to do so.

\[ \text{C}_{12} \text{H}_{16} \text{O}_{2} \text{NCl} \]

Calculated. N 7.09, Cl 17.97, neutral equivalent 197.5

Found. N 7.03, Cl 17.89, " 198

\textit{dl-Threonine Betaine Hydrochloride}—The crude product was purified in the same manner as the allothreonine derivative. Butyl alcohol was found to be a satisfactory solvent for recrystallization of the derivative, after it had been partially purified by one recrystallization from an alcohol-ether mixture. The yield of pure \textit{dl-threonine betaine hydrochloride} was 8 gm. (41 per cent).

\[ \text{C}_{12} \text{H}_{16} \text{O}_{2} \text{NCl} \]

Calculated. N 7.09, Cl 17.97, neutral equivalent 197.5

Found. N 7.17, Cl 18.04, " 199

\textit{dl-Threonine betaine hydrochloride} is considerably more soluble in absolute alcohol than the allothreonine derivative. The threonine betaine hydrochloride melts at 162–164°. A mixture of the isomers melts at 142–152°.
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Feeding Experiments

Albino rats weighing 125 to 175 gm. were used in the feeding experiments. The rats were maintained in separate metabolism cages and fed ad libitum. At the end of 21 days they were decapitated and the livers removed. The total fatty acid and unsaponifiable matter of the individual fresh livers were determined by the method of Leathes and Raper (5).

Each of the diets used contained Crisco 40, salt mixture (Osborne and Mendel (6)) 5, cod liver oil 1, the supplement, and glucose to make 100 per cent. The vitamin B factors were supplied in the form of two pills daily, each containing 75 mg. of milk concentrate and 10 mg. of fullers' earth adsorbate of a rice polishings

Table I
Effect of Various Substances on Production of Dietary Fatty Livers in Rats

<table>
<thead>
<tr>
<th>Supplement to diet*</th>
<th>Daily food intake</th>
<th>Change in weight</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per cent</td>
<td>gm. per cent</td>
<td>gm.</td>
</tr>
<tr>
<td>Casein</td>
<td>5.0</td>
<td>6.1</td>
<td>-14.3</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>5.0</td>
<td>5.5</td>
<td>-12.0</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>10.0</td>
<td>7.9</td>
<td>16.8</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>20.0</td>
<td>8.3</td>
<td>26.9</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>30.0</td>
<td>7.9</td>
<td>37.0</td>
</tr>
<tr>
<td>&quot; hydrolysate (7)</td>
<td>29.3</td>
<td>6.7</td>
<td>14.4</td>
</tr>
<tr>
<td>Tryptophane</td>
<td>0.7</td>
<td>5.1</td>
<td>-17.9</td>
</tr>
<tr>
<td>Casein</td>
<td>5.0</td>
<td>5.1</td>
<td>-17.9</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.25</td>
<td>5.1</td>
<td>-17.9</td>
</tr>
<tr>
<td>Casein</td>
<td>5.0</td>
<td>5.1</td>
<td>-17.9</td>
</tr>
<tr>
<td>Betaine hydrochlorides of</td>
<td>1.9</td>
<td>5.6</td>
<td>-17.7</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>1.9</td>
<td>5.5</td>
<td>-13.5</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>2.0</td>
<td>5.5</td>
<td>-11.7</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>2.0</td>
<td>5.5</td>
<td>-11.2</td>
</tr>
</tbody>
</table>

* Each experiment was carried out on a group of six rats (three males and three females) from the same litter.

† In these experiments, the term "lipid" is used to designate total fatty acid and unsaponifiable matter.

‡ Labco vitamin-free casein was used in all the experiments.
extract. The supplements, the concentrations at which they were incorporated in the diet, and the results of the feeding experiments are shown in Table I.

The results obtained with various levels of casein are similar to those reported by Channon and Wilkinson (2) and others. The basal diet (5 per cent casein) uniformly produced a highly fatty liver. A control group of rats receiving choline chloride showed no accumulation of fat in the liver.

The betaine hydrochlorides were fed at 5 times the molar level of the choline chloride. The rats showed no obvious ill effects from the ingestion of these quantities of the betaine hydrochlorides, and lost no more weight than the controls. Although there is considerable difference in the liver fat of the two groups of rats receiving serine betaine hydrochloride, the data show conclusively that the hydroxybetaines have no lipotropic effect.

Recently Welch and Welch (8) and Platt (9) have shown that choline has considerably more lipotropic effect than betaine. The former authors found that alanine betaine also has lipotropic activity but made no comparison of this substance with choline or betaine. These results combined with our data make possible a comparison of the lipotropic activity of choline chloride (I), betaine hydrochloride (II), serine betaine hydrochloride (III), and alanine betaine hydrochloride (IV). Such a comparison affords an interesting study of variation of physiological effect with structure in a group of closely related compounds. The results show that the introduction of a hydroxyl group into the betaine molecule may depress rather than enhance its effect on liver fat. Further, it is interesting to note that the introduction of a carboxyl group on the α-carbon of choline converts it into the lipotropically inactive serine betaine.

The mixture of amino acids obtained on hydrolysis of casein is as effective as casein in preventing the development of fatty livers in rats. Since the hydrolysate was practically phosphorus-
free, this result eliminates the possibility that the organic phosphorus of the casein is responsible for its lipotropic effect.

**SUMMARY**

1. A method has been developed for the synthesis of the betaine hydrochlorides of $dl$-serine, $dl$-threonine, and $dl$-allothreonine.

2. The betaines of $dl$-threonine and $dl$-allothreonine undergo a retrograde aldol condensation in an alkaline medium, yielding betaine and acetaldehyde.

3. The betaine hydrochlorides of $dl$-serine, $dl$-threonine, and $dl$-allothreonine do not prevent the development of a fatty liver in rats fed a high fat, low protein diet.

**BIBLIOGRAPHY**

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