L-CYSTATHIONINE IN HUMAN BRAIN

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During the course of the study of the distribution of N-acetylaspartic acid in brains of different species (1, 2), protein-free extracts of brain tissues were analyzed specifically for the aspartic acid derivative. In pursuing these investigations further, a survey was made by ion exchange chromatography of the concentration of all of the free acidic and neutral amino acids of the brain tissues, in a manner similar to that previously employed for study of the ninhydrin-positive constituents of the various tissues of the cat (3). The present investigation began when a striking difference was observed between the effluent curve obtained when an extract of human brain was analyzed and that found earlier (3) for cat brain. A major ninhydrin-positive peak was present in the region of the chromatogram between the valine and the methionine positions. Large amounts of material emerging at this effluent volume were not found either in the other brain extracts or in the other cat tissues previously analyzed. The present communication is concerned with the identification of the compound responsible for this peak on the chromatograms of the extracts of human brain.

EXPERIMENTAL

Preparation of Tissue Extracts—Protein-free extracts of the tissues were prepared with aqueous picric acid, and the picric acid was removed as described previously for rat brain (1). The human brains were obtained at autopsy; one or both occipital lobes were taken. Specimens of human liver, kidney, and muscle were also secured in one instance (death from arteriosclerotic cardiovascular disease). In addition to being indebted to those investigators previously mentioned (2), who generously provided samples of brains of different species, we are indebted to Dr. Joseph L. Melnick for cooperation in obtaining a monkey brain (rhesus).

Chromatographic Behavior of Unknown Compound—With the chromatographic system that has been employed with the automatic recording apparatus of Spackman, Stein, and Moore (4), the unknown compound appeared at 340 effluent ml. (Fig. 1). Qualitatively, the rest of the effluent curve was not markedly different from that obtained with cat brain. When the change to the buffer of pH 4.25 was not made, and the elution was continued with the sodium citrate buffer of pH 3.24, the unknown
compound was eluted at 514 ml., well separated from valine (at 344 ml.) and methionine (not eluted by 580 ml.). Thus, it seemed feasible to effect a good separation of the compound by the use of a single buffer on a shorter column. On a 0.9 × 15 cm. column of Dowex 50-X8 at 25°, use of a sodium citrate buffer at pH 3.35 as eluent afforded complete separation of the unknown compound, which appeared at 80 ml., just after the bulk of the acidic and neutral amino acids. This shorter column could be used as a rapid analytical method for comparing the amounts of the compound in different samples of human brain; it was scaled up, in modified form, for the following experiments directed toward the isolation of the substance.

Fig. 1. Chromatographic analysis of a protein-free extract of human brain on a 0.9 × 150 cm. column of Amberlite IR-120. A 0.2 N sodium citrate buffer of pH 3.24 was used as eluent for the first 250 ml., at which point elution was continued with a 0.2 N sodium citrate buffer of pH 4.25; both buffers contained 10 ml. of thiodiglycol and 5 ml. of BRIJ 35 per liter. The temperature was 50°. The curve is a tracing of an analysis carried out with the automatic recording apparatus (Spackman, Stein, and Moore (4)). The proline peak has been corrected for the relatively low color yield of this amino acid in order to bring the concentrations into approximately correct molar proportions relative to those of the other amino acids. An amount of extract from 0.4 gm. of brain tissue was chromatographed.
Isolation of Compound from Human Brain (Experiment I)—For the isolation of the compound, it was desired to use Dowex 50-X8 in the ammonium rather than the sodium form, with a volatile ammonium formate buffer as eluent (5, 6). Fig. 2 shows the effluent curve obtained when the extract from 190 gm. of human brain was fractionated on a 4 x 30 cm. column of Dowex 50-X8, with 0.2 M ammonium formate buffer of pH 3.32 (not containing thiodiglycol or BRIJ 35) as eluent. For the ninhydrin analyses, 0.2 ml. aliquots were withdrawn from every third 12 ml. fraction, and the ammonium salts were removed at 50° in a vacuum desiccator (5).

Under these conditions, a second peak followed the main one and indicated the presence of an overlapping component which did not emerge at this position when the eluent was sodium citrate. The effluent fractions comprising the A and B zones were pooled as indicated in Fig. 2. Each portion was separately freed of buffer by concentration on a rotary evap-
orator and two lyophilizations. Recoveries were as follows: Fraction A, 108 mg. of a slightly yellow powder; Fraction B, 181 mg. of a white powder. The observed weights and the total ninhydrin colors of the two fractions indicated the presence, particularly in Fraction B, of one or more substances possessing a relatively low color yield in the ninhydrin reaction. It was noted, further, that Fraction A was much less soluble in water than Fraction B.

Part of Fraction A (21 mg.) was dissolved in a minimal amount of hot water and allowed to crystallize overnight in the cold; yield, 6 mg. (Fraction A-1). The filtrate was concentrated and precipitated with ethanol to yield, after standing overnight in the cold, 8 mg. of crystals (Fraction A-2). Infrared spectra¹ of Fractions A-1 and A-2 indicated that they were different substances. When the infrared spectrum of Fraction B was determined, it was found to be identical with that of Fraction A-2.

Part of Fraction B was recrystallized from water and submitted to analysis. Ash = 0.0 per cent.

C₄H₅NaO₂ (131.1). Calculated. C 36.63, H 6.92, N (Dumas) 32.07
Found. " 36.72, " 6.96, " " 32.17

The substance gave an analysis fitting that of creatine, and, in addition, the infrared spectrum of a known sample of creatine was identical with that of Fractions B and A-2.

To remove the contaminating creatine from the major part of Fraction A, the remainder of the material (87 mg.) was dissolved in 8 ml. of hot water and cooled slowly to room temperature. The crystals that formed were removed by filtration and washed with water; yield, 6 mg. (Fraction A-3). The filtrate and washings were concentrated to 5 ml. and cooled to room temperature; 11 mg. of yellow crystals were deposited (Fraction A-4), and it appeared at this stage that the mother liquors probably contained at least one principal (and probably ninhydrin-negative) contaminant in addition to creatine. A further concentration caused a deposition of white crystals, which, however, became admixed with yellow crystals after standing in the cold overnight; yield, 12 mg. (Fraction A-5).

The first of the above three crops of crystals (Fraction A-3) was submitted to analysis. Ash = 0.1 per cent.

C₁₇H₁₅N₄O₈S (222.3). Calculated. C 37.83, H 6.35, N (Dumas) 12.60
Found. " 37.89, " 6.44, " " 12.83

Insufficient material was available for a sulfur analysis, but the presence of sulfur was suspected from the behavior of the substance in the C and H

¹ It is a pleasure to acknowledge the generous cooperation and assistance of Dr. Herbert Jaffe in the determination of the infrared spectra reported in this paper.
determination. From the empirical formula, the substance could have been either cystathionine or β-methyllanthionine. The fact that the former is known to emerge from columns of Dowex 50-X4 at a position between valine and methionine (7) was consistent with the supposition that the unknown might be cystathionine. The infrared spectrum obtained initially from synthetic L-cystathionine, though containing the same absorption bands as the spectrum of Fraction A-3, showed a different intensity of absorption. However, when the synthetic amino acid, which had originally been crystallized from a salt solution (8), was recrystallized from water, as the isolated sample had been, the infrared spectrum became identical with that of Fraction A-3.

Elemental analysis of the impure Fraction A-4 showed that it had a higher content of C and N and a lower content of H than cystathionine. In an attempt to ascertain the nature of the impurity, the mixed crystals of Fraction A-5 were separated manually under a magnifying glass into white crystals, 8 mg. (Fraction A-5-a), and yellow crystals, 4 mg. (Fraction A-5-b). The white crystals gave the infrared spectrum of cystathionine. In order to account for the high N and low H content of Fraction A-4, it was thought that the yellow crystals might be a purine or a pyrimidine. The ultraviolet absorption spectrum of an aqueous solution of Fraction A-5-b was, therefore, determined on a Beckman DU spectrophotometer. Maximal absorption occurred at a wave length of 250 mμ, and the absorbance ratios at different wave lengths were as follows: 250:260 mμ, 1.37; 280:260 mμ, 0.07; 290:260 mμ, 0. These values suggested that the compound was hypoxanthine (cf. Beaven et al. (9)). A comparison of the infrared spectrum of synthetic hypoxanthine with that of Fraction A-5-b showed almost complete identity, although there were several minor peaks in the spectrum of Fraction A-5-b that coincided with major absorption maxima in the cystathionine spectrum.

Isolation of Cystathionine from Human Brain (Experiment II)—The experiments described above, which led to the isolation of the unknown compound from human brain and its characterization as cystathionine, showed the first preparation to be contaminated by creatine and hypoxanthine which were not completely removed by the relatively short column chosen for the preparative chromatography. The following simpler method of isolation was therefore adopted in order to obtain purer material. The protein-free extract, prepared in the usual manner, was made 1 N with respect to HCl by the addition of concentrated HCl. The acidified solution was heated for 1 hour under reflux (bath temperature, 120°)

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2 We are greatly indebted to Dr. Marvin D. Armstrong for generous samples of synthetic L-cystathionine and L-allo-cystathionine.

3 We are indebted to Dr. Elliott N. Shaw for a sample of synthetic hypoxanthine.
in order to convert creatine to creatinine; the solution was then cooled, filtered, and taken to dryness three times to remove HCl. The final residue was dissolved in water, brought to pH 2 with a few drops of concentrated NH₄OH, and chromatographed on Dowex 50-X8. The results were similar to those shown in Fig. 2, except that the ninhydrin-positive shoulder on the cystathionine peak was absent, and this region contained no creatine, as determined by the Jaffe reaction on the combined effluent fractions after boiling the solution with 1 N HCl.

In order to separate the cystathionine from hypoxanthine chromatographically, the combined effluent fractions from the peak were first freed of buffer by concentration on a rotary evaporator and two lyophilizations. The residue was dissolved in ammonium formate buffer of pH 2.8 and chromatographed in two portions on a 2 X 15 cm. column of Dowex 50-X8 in the ammonium form, with 0.2 M ammonium formate buffer of pH 3.00 as eluent. Under these conditions, hypoxanthine emerges at 250 effluent ml. and cystathionine at 517 ml.

The fractions containing cystathionine were combined and freed of buffer by concentration and lyophilization, and the residue was dissolved in water and filtered through a pad of Celite to remove fibers and particles of resin. The filtrate and washings were concentrated and cooled to 5°; the crystals that formed were filtered, washed with cold water, and dried in vacuo over P₂O₅. Yield, 22.2 mg. Ash = 0.1 per cent.

\[ C_7H_{14}N_2O_4S \ (222.3) \]

Calculated. C 37.83, H 6.35, N (Dumas) 12.60, S 14.43

Found. " 37.90, " 6.48, " 12.69, " 14.06

\[ \text{Fig. 3. Infrared absorption of L-cystathionine in KBr, determined with a Perkin-Elmer model 21 double beam spectrometer with NaCl optics, set at a scanning speed of 0.2 μ per minute on a chart scale of 5 cm. for 1 μ. The sample of synthetic L-cystathionine was crystallized from distilled water.} \]
[\alpha]_D^{25} +26.4^\circ (0.8\ \text{per cent in } N\ \text{HCl}),\text{ determined with a Beckman DU spectrophotometer fitted with a polarimetric unit (model D, series No. 112, Standard Polarimeter Company, New York). With the same apparatus, a sample of synthetic L-cystathionine gave [\alpha]_D^{25} +28.3^\circ (0.8\ \text{per cent in } N\ \text{HCl});\text{ the value given by Armstrong (8) is } [\alpha]_D^{25} +23.9^\circ.\text{ Horowitz (10) reports } [\alpha]_D^{25} +26^\circ \pm 2^\circ \text{ for natural L-cystathionine.}

The infrared spectra of the isolated cystathionine and of the synthetic material after recrystallization from water are given in Fig. 3.

**Table I**

**Concentration of Cystathionine in Various Animal Tissues**

The chromatographic analyses were performed on aliquots of a picric acid extract with an automatic recording apparatus (4). For the human and monkey brains, the occipital lobes were taken; for the other human tissues, random portions; for the cow brain, random portions of the cerebrum; and, in the other cases, whole brains or combined brains (the number is given in parentheses) were used. The five human brains were from individuals dying of cancer of the pancreas, Wilson's disease, leucemia, arteriosclerotic cardiovascular disease, and myocardial infarction, respectively.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Tissue</th>
<th>Cystathionine concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man</td>
<td>Brain</td>
<td>56.6</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>55.4</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>55.1</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>25.6</td>
</tr>
<tr>
<td></td>
<td>&quot;</td>
<td>22.5</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>0.8</td>
</tr>
<tr>
<td>Monkey</td>
<td>Brain</td>
<td>12.8</td>
</tr>
<tr>
<td>Cow</td>
<td>&quot;</td>
<td>1.2</td>
</tr>
<tr>
<td>Cat</td>
<td>&quot;</td>
<td>2.7</td>
</tr>
<tr>
<td>Rat</td>
<td>&quot;</td>
<td>3.9</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>&quot;</td>
<td>3.1</td>
</tr>
<tr>
<td>Chicken</td>
<td>&quot;</td>
<td>0.6</td>
</tr>
<tr>
<td>Duck</td>
<td>&quot;</td>
<td>0.2</td>
</tr>
<tr>
<td>Horseshoe crab</td>
<td>Brains (2)</td>
<td>15.0*</td>
</tr>
</tbody>
</table>

* The extract of the horseshoe crab tissue contained about 10 times the amounts of the common amino acids found in the other brains, and cystathionine was thus not high relative to the other amino acids.

**Distribution of Cystathionine in Animal Tissues**—Extracts of the brains of various species and extracts of several human tissues were analyzed by

* We are greatly indebted to Dr. Lyman C. Craig, who generously measured the optical rotations with the spectrophotometric equipment.
using 0.9 × 150 cm. columns with the automatic recording apparatus (4) (see Fig. 1). The results are presented in Table I. The largest amounts of cystathionine were found in human brain, but detectable quantities of material emerging exactly at the cystathionine position were found in extracts of other brains and in the other human tissues examined. It should be emphasized, however, that, except in the case of the human brains, the identification of the compound is based solely on its position on the chromatogram and that the results shown in Table I must therefore be considered as maximal figures.

The observed value of 15.0 mg. per cent cystathionine in the brain of the horseshoe crab (Table I) has to be considered in conjunction with the fact that in this tissue all of the other amino acids were more concentrated by about a factor of 10 than they are in human brain. Relative to the other amino acids in the crab brain, cystathionine was not present in a higher concentration than it is in the brains of most of the other species studied.

**DISCUSSION**

The formation of cystathionine in the metabolic conversion of methionine to cysteine has been clearly established; the work has been reviewed by du Vigneaud (11). The existence of the compound in nature was demonstrated by Horowitz (10), who isolated it from the mycelium of a methionineless mutant of *Neurospora*, and by Hess (12) and Tabachnick and Tarver (13), who showed chromatographically that it was present in fasted rats fed methionine and serine. Berg (14) found that cell-free extracts of pigeon liver catalyzed the anaerobic incorporation of formate-C\(^{14}\) into cystathionine. However, to our knowledge, cystathionine has not heretofore been shown to be present in measurable quantities as a normal constituent of a mammalian tissue.

That the conversion of methionine to cysteine actually takes place in brains has been shown by Gaitonde and Richter (15), who used S\(^{35}\)-labeled methionine. The demonstration in the present work of the occurrence of cystathionine in relatively large amount in extracts of human brain suggests the existence in this tissue of a special relationship between the amounts of the enzymes involved in the synthesis and in the cleavage of the amino acid. One possibility is that the cystathionase of human brain, an enzyme not yet studied, is extremely labile. Were this the case, in

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\(^5\) The short 0.9 × 15 cm. columns referred to earlier can be used for the analysis of human brain, but the relatively broad peak does not provide an accurate estimate of the amount of cystathionine present when the concentration is as low as it is in most brain tissues other than the human. The ninhydrin color yield from cystathionine (per two NH\(_2\) groups) is 1.20.
the interval between the death of the individual and the treatment of the brain sample with picric acid, cystathionine might continue to be formed, but, not being broken down in the normal manner, would accumulate. Alternatively, it is possible that in human brain the concentration of cystathionase is such that, at equilibrium, there is a large amount of cystathionine present, although the process of sulfur transfer otherwise proceeds in the usual manner. Other explanations would require that the human brain differ in its sulfur metabolism from the other tissues of the human body and from the brains of other species.

The question of vitamin B₆ deficiency also bears on the subject of cystathionine metabolism. Cystathionase has been reported to be a pyridoxal-dependent enzyme (Binkley et al. (16)), and Blaschko and Hope (17) have found that a pyridoxine deficiency in the rat results in the excretion of cystathionine, presumably as a result of a metabolic block leading to its accumulation. Since, of necessity, the human brains used in the present study were not obtained from "normal" subjects, the effect of a possible vitamin B₆ deficiency on brain cystathionase would have to be investigated.

With regard to the isolation of creatine and hypoxanthine in the course of the present work, it should be noted that the occurrence of both compounds has long been known. Creatine was isolated from human brains in 1857 by Müller (cf. IIunter (18)), and the presence of hypoxanthine was reported by Thudicum in 1884 (cf. Page (19)).

SUMMARY

L-Cystathionine has been found in protein-free extracts of five human brains at concentrations ranging from 22.5 to 56.6 mg. per 100 gm. wet weight of tissue. Ion exchange columns have been used for the isolation of the compound in quantity sufficient to permit characterization by elementary analysis, optical rotation, and infrared spectrum. Analytical scale chromatograms have shown human liver, kidney, and muscle to contain less than 1 mg. per cent of cystathionine. The concentration in the brains of other species was less than 4 mg. per cent, except in the brain of the rhesus monkey, which contained 12.8 mg. per cent, and in the special case of the brain of the horseshoe crab (15 mg. per cent), in which the concentrations of nearly all of the accompanying amino acids were nearly 10-fold those found in the brain tissues of the other species studied.

It is a pleasure to acknowledge the cooperation of Dr. Darrel H. Spackman, as well as the technical assistance of Miss Kerstin Johansson and Miss Aileen M. Cordon, in carrying out the amino acid analyses on the automatic recording apparatus. We are greatly indebted to Mr. S.
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