ACKNOWLEDGMENT

The authors thank Henry L. F. Vix for supplying the seed and Jack Berquist for the photography.

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Received for review June 12, 1972. Accepted November 3, 1972. Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

Stachydrine: Content in Alfalfa and Biological Activity in Chicks

Margaret A. Connor,* J. Benjamin Stark, James C. Fritz,[1] and George O. Kohler

Stachydrine (proline betaine) has been identified as the major quaternary nitrogen base in alfalfa. Its content in dehydrated alfalfa meals has been positively correlated with protein contents. More stachydrine was present in the leaves than in the stems. A chick feeding study showed that stachydrine decreased the incidence of perosis.

Some confusion has existed over the identity of the quaternary nitrogen bases present in alfalfa. Steenbock (1918) first isolated and identified stachydrine (proline betaine) as a component of alfalfa hay, and later Vickery (1925) found that it was the principal quaternary base present in alfalfa. However, a recent report, "A Study of the Major Nutritional Constituents of Dehydrated Alfalfa" (American Dehydrators Association, 1965), lists the major base to be betaine (glycine betaine). Due to the possible physiological differences of the two bases, studies were conducted in this laboratory to identify the quaternary bases present, to determine which are the major constituents, to develop a satisfactory method for their determination, and to examine their biological activity.

EXPERIMENTAL SECTION

Identification of Quaternary Ammonium Compounds Present in Alfalfa. A partially deproteinated pressed juice from fresh alfalfa, concentrated to 54.2% solids (Bickoff *et al.*, 1968), was fractionated according to the ion-exchange procedure of Stark (1962). The fraction containing the quaternary ammonium compounds was collected and concentrated (organic base concentrate).

A 95-mg sample of this organic base concentrate in 2.5 *N* HCl was fractionated on Dowex 50W (200–400 mesh), according to the procedure of Christianson *et al.* (1960). The order of elution and extent of separation of stachydrine, betaine, and choline were determined by chromatography of control materials.

Four 500-ml fractions and one 1-l. fraction of effluent were scanned for quaternary nitrogen compounds by measurement of the ultraviolet absorption of the periodide derivative (Wall *et al.*, 1960). Further identification was carried out by thin-layer chromatography on silica gel/Kieselguhr (25/75 by weight) using water (100%) or ethanol–ammonia (95:5) as the developing solvent systems. A modified Dragendorff reagent (Bregoff *et al.*, 1953) was used for detection. Identification of bands was carried out by comparison of R_f values of known samples of choline, stachydrine, betaine, and trigonelline (betaine of *N*-methyl nicotinic acid).

Trigonelline in the organic base fraction was determined using the spectrophotometric procedure of Moores and Greninger (1951).

Routine Procedure Developed for Determination of Stachydrine in Alfalfa. An ion-exchange procedure was used to separate choline and stachydrine as follows. Dehydrated alfalfa (10 g) was blended with 500 ml of 80° water in a blender for 15 min. Approximately 10 g of Celite was added and the extract was filtered with suction. The filter cake was washed three times with 100-ml portions of water. Water was added to the combined filtrate to reach a final volume of 1 l. and 500 ml was passed through a 20-cm^3 column of Dowex 50 (H+) X-8, 50–100 mesh resin. The resin was washed with 60 ml of water and eluted with 100 ml of 1 *N* ammonium hydroxide, followed by 40 ml of water applied in small aliquots. The combined ammonia eluate and subsequent washings were evaporated on a rotary evaporator to remove free ammonia and the remaining solution was acidified with 2 ml of 6 *N* HCl and made to 100 ml with water. Analyses were carried out using the method of Focht *et al.* (1956). Concentration was estimated from a standard curve prepared from stachydrine–HCl reineckate.

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Table I. Composition of Basal Diets[a]

Ingredient	Semipurified diet, %	More practical diet, %
Glucose monohydrate	51.85	
Degermed yellow corn meal		50.85
Corn gluten meal	15.00	20.00
Lactalbumin	5.00	
Dehulled soybean meal	5.00	10.00
Casein	5.00	
Gelatin	5.00	
Wheat middlings		5.00
Blood meal	5.00	4.00
Corn oil	5.00	4.00
Alfalfa meal		2.50
Dicalcium phosphate	2.50	2.00
Calcium carbonate		1.00
Iodized salt	0.50	0.50
Vitamin premix[b]	0.10	0.10
Trace mineral mix (Dawe's)	0.05	0.05

[a] In addition, 0.5 mg of menadione was added per kg of the semipurified diet. No vitamin K supplement (except the alfalfa) was added to the more practical diet. The more practical diet was estimated to contain 776 mg of choline chloride/kg or about twice as much as the semipurified diet. [b] Vitamin premix: vitamin A, beadlets (500,000 I u/g) 1.500 g; vitamin D_3, dry mix (200,000 ICu/g) 1.200 g; vitamin E, 25% beadlets 3.200 g; riboflavin, 0.500 g; calcium pantothenate, 0.900 g; niacin, 4.500 g; thiamine hydrochloride, 0.500 g; pyridoxine hydrochloride, 0.500 g; folic acid, 0.075 g; vitamin B_{12}, 0.1% dry mix 3.300 g; and glucose, 133.825 g.

To determine the precision of the extraction method, nine 10-g portions of a dehydrated alfalfa sample (20% protein grade, 6.88% moisture) were analyzed by the preceding method. The stachydrine values of the nine replicate samples ranged from 73.9 mg/10 g of alfalfa to 80.9 mg/10 g, with an average of 77.4. Standard deviation is 2.10.

The percentage recovery of added stachydrine was measured by adding 79.7 mg of stachydrine (99.6 mg of stachydrine-HCl) to each of three 10-g replicate samples of dehydrated alfalfa and measuring the total amount of stachydrine. The average value of 77.4 mg of stachydrine obtained in the previous experiment was taken as the amount occurring naturally. Results showed values of 160.0, 157.4, and 163.2 mg of stachydrine/10 g sample. This represents an average recovery of 101% of added stachydrine.

Chick Feeding Study. The biological activity of stachydrine as a methyl donor was measured by a feeding study in which 20 groups of chicks (day-old, straight run White Rocks) were fed two basal diets supplemented with choline or related compounds. The compositions of the two basal diets are listed in Table I.

Ten chicks were started in each of groups 1, 2, and 5. Nine chicks were started in each of the other groups. Supplements were stachydrine hydrochloride, choline chloride, fish protein concentrate, betaine hydrochloride, carnitine hydrochloride, and *dl*-methionine, in addition to a control with no supplement. After 3 weeks on the supplemented diets, the chicks were weighed and given a perosis score. The data were analyzed using the Duncan's multiple range test (1955).

RESULTS AND DISCUSSION

Identification of Quaternary Ammonium Compounds Present in Alfalfa. The results of ion exchange chromatography and thin-layer analysis of the organic base concentrate of alfalfa showed stachydrine to be the major component. Choline, present in the original alfalfa juice concentrate, was not found in the organic base fraction. Choline behaves more like a sodium or potassium ion on a strong cation exchanger and was not eluted by ammonia in the procedure for separation of the quaternary ammonium components of alfalfa. This property of choline permitted its ready separation from stachydrine through ion-exchange fractionation and is the basis for the routine procedure developed for the determination of stachydrine in alfalfa. This procedure is satisfactory when either betaine or stachydrine is present as a single component.

The fraction of the organic base concentrate predicted from the elution curves to contain betaine was analyzed for quaternary ammonium compounds. Stachydrine was the only one detected. No betaine was found even after a 100-fold concentration of the fraction. In a prior control run, betaine in excess of 1% of the total quaternary compounds could be detected. Therefore betaine is assumed not to be present in alfalfa in a quantity of more than 1% of the total stachydrine.

The fraction predicted to contain stachydrine showed stachydrine as the only quaternary ammonium compound present. No concentration was necessary for detection of stachydrine by thin-layer chromatography. Estimation of the amount by the periodide procedure showed 26.0 mg of stachydrine per 95 mg of organic base concentrate, the amount of concentrate put on to the column. This is in agreement with the analysis of the original material, which showed a total of 24.8 mg per 95 mg of organic base concentrate, calculated as stachydrine.

Thin-layer analysis of the final fraction, concentrated 300-fold, indicated that both stachydrine and trigonelline were present. Quantitative estimation of trigonelline indicated an amount of less than 2% of the original organic base fraction.

The values of stachydrine in the tables include the small contribution due to trigonelline.

Results of Routine Procedure for Determination of Stachydrine in Alfalfa. Samples of four commercial

Table II. Stachydrine Content of Commercial Grades of Alfalfa

Sample[a]	Protein[b,c] grade	% protein[c,d]	Quaternary bases calculated as betaine[b,d]		Stachydrine[d]	
			mg/100 g of sample	% N of total N	mg/100 g of sample	% N of total N
1[e]	15	16.3	502	2.3	644	2.4
1[f]					656	2.5
2[e]	17	19.4	517	2.0	679	2.1
2[f]					793	2.2
3[e]	20	22.2	601	2.0	793	2.2
3[f]					831	2.3
4[e]	22	24.2	585	1.8	795	2.0
4[f]					767	1.9

[a] References *e* and *f* refer to duplicate subsamples of lots 1 to 4, respectively. [b] American Dehydrators Association's (1965) report. [c] N × 6.25. [d] Moisture-free basis.

Table III. Stachydrine Content of Hand-Separated Leaf and Stem Fractions of Freeze-Dried Alfalfa[a]

No.[b]	Alfalfa sample	Stachydrine, mg/100 g of sample	% protein (N × 6.25)	Stachydrine, % N of total N
2	Stem	830	13.8	3.7
2	Leaf	1180	35.5	2.0
4	Stem	630	11.6	3.4
4	Leaf	1270	34.2	2.3
5	Stem	640	13.1	3.0
5	Leaf	930	33.2	1.7
10	Stem	740	13.1	3.5
10	Leaf	1150	33.2	2.1
11	Stem	680	15.4	2.7
11	Leaf	790	33.1	1.5
A	Stem	340	11.6	1.8
A	Leaf	770	31.3	1.5

[a] Moisture-free basis. [b] Sample numbers correspond to those described in Livingston *et al.* ('968b) (June harvest). Sample A was not included in that report.

grades of dehydrated alfalfa 15, 17, 20, and 22% protein were collected, blended, and subsampled by the Midwest Research Institute for the American Dehydrators Association to obtain comprehensive analyses for use in feed formulation. These samples were analyzed for betaine by a commercial laboratory using the reineckate procedure of Beattie (1936), which was modified by the Wisconsin Alumni Research Foundation and was described in the U. S. Department of Agriculture Technical Bulletin 1235 (Binger *et al.*, 1961). Aliquots of the samples were obtained and analyzed for stachydrine. The results of the analyses are shown in Table II. It is apparent that the procedure used for the determination of betaine was in fact determining stachydrine. The amount of stachydrine increases with increasing percent of protein. The correlation coefficient between stachydrine content and percent of protein for the samples listed in Tables II and III was calculated to be 0.792. This value is significant at the 1% probability level.

Due to the higher molecular weight and more intense color of the reineckate salt precipitates, stachydrine values are higher than estimations made on the basis of betaine. When the stachydrine values are multiplied by 0.75, they agree very closely with the betaine values in the American Dehydrators Association's (1965) report. The stachydrine in the alfalfa samples accounted for about 2 to 2.5% of the total nitrogen content.

Stachydrine analyses were carried out on hand-separated leaf and stem fractions of freeze-dried alfalfa samples, originally prepared for xanthophyll studies (Livingston *et al.*, 1968b). Results are tabulated in Table III.

It is evident from the data that there is a greater amount of stachydrine present in the leaves than in the stems. However, the stachydrine present in the stems represents a greater percentage of the total nitrogen.

Stachydrine analysis of whole alfalfa, dehydrated in an Arnold alfalfa dehydrator (Livingston *et al.*, 1968a) at an outlet temperature of 320°F, showed essentially no loss of stachydrine when compared with the same alfalfa lyophilized fresh. A value of 930 mg of stachydrine/100 g of moisture-free sample was found for the lyophilized alfalfa and 960 mg/100 g of moisture-free sample was found for the dehydrated alfalfa, indicating that stachydrine is not destroyed by the heat of dehydration.

Feeding Study. Results of the chick feeding study of two basal diets supplemented with choline and related compounds are shown in Tables IV and V. Under the con-

Table IV. Effect of Choline and Related Compounds on Growth and Perosis of Chicks Fed Semipurified Diet[a]

Group number	Supplement	Average weight, g at 3 weeks	Average perosis score[b]
1	None	221 c	3.0 a
2	Choline chloride, 300 mg/kg	294 b	1.95 b
3	Choline chloride, 600 mg/kg	354 a	1.78 b
4	Choline chloride, 900 mg/kg	368 a	0.58 c
5	Fish protein concentrate, 5%	253 bc	2.44 ab
6	Betaine hydrochloride, 660 mg/kg	242 c	2.44 ab
7	Stachydrine hydrochloride, 1158 mg/kg	223 c	2.15 b
8	Carnitine hydrochloride, 1274 mg/kg	235 c	2.39 ab
9	Carnitine hydrochloride, 849 mg/kg	220 c	2.56 ab
10	*dl*-Methionine, 1926 mg/kg	261 bc	2.28 ab

[a] Means bearing the same letter do not differ significantly ($\alpha = 0.05$), Duncan's test. [b] Perosis scoring system: 0 = normal legs; 1 = slight enlargement of hock joints; 2 = marked enlargement of hock joints, with some bending and shortening of the long bones of the leg; 3 = severe bending of the long bones; 4 = tendon slipped off hock joints, chick able to move about only on its hock joints.

Table V. Effect of Choline and Related Compounds on Growth and Perosis in Chicks Fed Practical Diet[a]

Group number	Supplement	Average weight, g at 3 weeks	Average perosis score[b]
1	None	340 a	0.95 abc
2	Choline chloride, 200 mg/kg	331 a	0.56 bc
3	Choline chloride, 400 mg/kg	328 a	0.39 d
4	Choline chloride, 600 mg/kg	331 a	0.44 c
5	Fish protein concentrate, 5%	395 a	1.00 ab
6	Betaine hydrochloride, 440 mg/kg	332 a	0.89 abc
7	Stachydrine hydrochloride, 772 mg/kg	321 a	0.79 abc
8	Stachydrine hydrochloride, 1158 mg/kg	344 a	0.38 d
9	Carnitine hydrochloride, 566 mg/kg	312 a	0.67 abc
10	*dl*-Methionine, 1284 mg/kg	325 a	1.10 a

[a] Means bearing the same letter do not differ significantly ($\alpha = 0.05$), Duncan's test. [b] Perosis scoring system: 0 = normal legs; 1 = slight enlargement of hock joints; 2 = marked enlargement of hock joints, with some bending and shortening of the long bones of the leg; 3 = severe bending of the long bones; 4 = tendon slipped off hock joints, chick able to move about only on its hock joints.

ditions of the chick feeding study, choline chloride at all three levels was significantly effective in both increasing growth and preventing perosis on the semipurified basal diet and in decreasing perosis at the 400 mg/kg level in the practical diet. Although stachydrine did not effect a growth response, it did produce a significant decrease in the incidence of perosis at the 1158 mg/kg level in both diets. Betaine and methionine did not prevent perosis to a statistically significant degree.

In studying the various responses of methyl donors in animals, Moyer and du Vigneaud, (1942) fed to rats an amino acid diet devoid of methionine and cystine but containing homocystine and vitamin supplements. On this diet, choline chloride increased growth. Betaine, when added to a similar diet (Chandler and du Vigneaud, 1940; du Vigneaud *et al.*, 1939), increased growth, but choline was more effective than betaine. Jukes and Stokstad (1952) obtained similar results on low vitamin B_{12} diets with and without vitamin B_{12} additions.

In looking at the problem of perosis, Jukes (1940) found that choline, but not betaine, prevented perosis in turkeys. The diet fed was not deficient in methionine or cystine.

In studying compounds acting as methyl donors in man, Ciusa and Nebbia (1948) identified stachydrine, choline, betaine, and several other compounds as effective methyl donors.

The diets used in this present study meet NRC requirements of methionine and do not show great differences in growth response among the supplements. In fact, in the practical type basal diet, the average weight in each supplemented group did not differ statistically from the control. However, choline and stachydrine both effected a decrease in the incidence of perosis. Jukes (1971) has discussed the question of why betaine and methionine are ineffective as substitutes for choline in preventing perosis in chicks and turkeys. He suggests that choline may function directly in bone formation as opposed to functioning as a precursor of betaine and methionine in the prevention of perosis. The results of our study indicate that stachydrine decreases the incidence of perosis, but no mechanism can be postulated at this time.

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Received for review May 4, 1972. Accepted October 26, 1972. Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

Flavor Compounds: Volatilities in Vegetable Oil and Oil-Water Mixtures. Estimation of Odor Thresholds

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Air to vegetable oil partition coefficients have been determined experimentally for a number of organic flavor compounds. These are shown to be, in general, of the same order as values calculated from solution-vapor theory, assuming that their activity coefficients in vegetable oil are equal to 1. A simple method of calculating the volatilities (air to mixture partition coefficients) of compounds in vegetable oil-water mixtures is derived and shown to compare reasonably well with experimental results for such mixtures. A method is also developed for calculating odor thresholds for compounds in vegetable oil solutions from their known thresholds in water solutions. This is shown to give values which are of the same order as experimentally determined values.

The authors are carrying out a continuing study of the aroma and flavor of fried foods (*cf.* Buttery and Ling, 1972; Guadagni *et al.*, 1972). An important factor in the effectiveness of various aroma compounds in foods is their volatility in the food medium. This is controlled to a considerable extent by the affinity of the compounds for the particular medium(s) in the food.

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With complex systems such as we have in foods, it is useful to have some model systems to relate to. Studies of these model systems can at least give us an approximation of the behavior we might expect in the actual practical system. The authors have previously studied the volatilities of a number of flavor compounds in a pure water medium (Buttery *et al.*, 1969, 1971). This could be considered as one type of model system. A second important model system with foods could be the volatilities of various flavor compounds in vegetable oil. A third model system could be that for water-vegetable oil mixtures. Some