Purine Derivatives in Alfalfa as Growth Stimulants for *Bacillus subtilis*

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An aqueous extract of fresh alfalfa has been shown to stimulate the growth of microorganisms. A procedure for isolation of the active factors was employed which involved fractionation by ion exchange, countercurrent distribution, and paper chromatography. A microbiological assay using *Bacillus subtilis* (ATCC 6633) was used to follow the activity during the fractionation. Adenine, guanine, adenosine, guanosine, hypoxanthine, xanthine, inosine, isocytosine, and cytidine were the factors which stimulated growth of the microorganism. Quantitative determinations of the three most abundant compounds (adenine, adenosine, and guanosine) have shown them to be present in sufficient quantities to account for almost all of the activity. The other compounds were present in trace amounts.

Alfalfa is a valuable ingredient in most animal feeds because of its abundance of proteins, carbohydrates, vitamins, and minerals. There is a great volume of published literature reporting that fresh forages such as alfalfa are rich sources of unidentified factors which beneficially affect growth, health, and reproductivity of poultry, swine, ruminant animals, guinea pigs, and rabbits (Kohler, 1953; Rasmussen, 1965; Liuzzo *et al.*, 1960; Lakhanpal *et al.*, 1966). One of the first growth promoters reported to be present in forages was the "grass-juice factor" (Kohler *et al.*, 1938; Kohler and Graham, 1951) for guinea pigs, rats, and chicks. Scott (1951) obtained growth responses with alfalfa juice on turkeys.

Because of the large quantities of material and considerable time required for animal assays, a rapid, microbiological assay would be a more satisfactory method for following the purification of unidentified growth-stimulating factors obtained from fresh alfalfa juice. The possibility that a microorganism response can correlate with an animal response is well documented in the literature. For example, folic acid, when still an unidentified factor, was known as the eluate factor for *Lactobacillus casei*, as vitamin M for monkeys, as Factor U and vitamin B_c for chicks, as Wills anemia factor for human beings, and as folic acid for *Streptococcus lactis*. Vitamin B_{12} also was sought for a number of years by many workers as the LLD factor for *Lactobacillus lactis*, as the antipernicious anemia factor for humans, as the animal protein factor for chicks, dogs, and pigs, as the manure factor for chicks, as "Factor X" and as Zoopherin for rats. More recently this approach is being explored by Novak and co-workers, who have reported the preparation of an acid extract of alfalfa which is active for *Neurospora sitophila* (Novak *et al.*, 1958), chicks (Liuzzo *et al.*, 1960), and thyroid-stressed rats (Lee *et al.*, 1961b).

Since Wisconsin Alumni Research Foundation (WARF) had a large number of organisms on hand and facilities for rapid screening, a contract was arranged whereby they would evaluate a variety of organisms for degree of response to an alfalfa juice extract prepared in the manner described for the grass-juice factor (Kohler and Graham, 1951). These studies showed that a number of microorganisms and insects respond to the addition of this extract to their normal basal diet by exhibiting an enhanced rate of growth. *Bacillus subtilis* (ATCC 6633) showed the most promise as a test organism for a microbiological assay to guide the isolation of stimulatory factors from alfalfa.

EXPERIMENTAL

Screening. The preliminary screening procedure used by WARF was as follows: Paper disks saturated with alfalfa juice extract were observed for growth stimulation on agar plates using a medium which supported growth of the organism being tested. About 40 organisms, including 19 bacteria, were screened. *Leuconostoc citrovorum*, *Bacillus polymyxa*, *Bacillus megaterium*, and *Bacillus subtilis* gave good growth responses. Several molds and actinomycetes, including *Fusarium oxysporum* and *Alternaria solani*, also gave good responses. Of the insects tested, the housefly gave the best response. The oyster mushroom (*Pleurotus ostreatus*) was the only fungus sensitive to the alfalfa extract.

Culture. The *B. subtilis* spores (ATCC 6633) were obtained from Difco Laboratories as an aqueous suspension. Assays requiring spores were inoculated with 0.05 ml. per tube of this prepared spore suspension. For assays requiring vegetative cells, the inoculum was prepared by inoculating 5 ml. of medium B (Table I) and 5 ml.

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Table I. Composition of Media in Grams per Liter

Component	Medium A	Medium B
Dextrose	100	5
Casein hydrolyzate[a]	10	10
Potassium citrate	10	10
Citric acid	2	2
KH_2PO_4	1.12	1.12
Salts:		
KCl	0.864	0.864
$CaCl_2$	0.254	0.254
$MnSO_4 \cdot H_2O$	0.005	0.005
$FeCl_3 \cdot 6H_2O$	0.005	0.005
$MgSO_4 \cdot 7H_2O$	0.254	7.680
Vitamin solution,[b] ml.	100	100
Temperature, ° C.	29	35
pH	5.4	7.0

[a] Nutritional Biochemical Co. (acid hydrolysis).
[b] Vitamin solution per liter: Ca pantothenate, 50 mg.; niacin, 50 mg.; thiamine, 5 mg.; biotin, 0.5 mg.; vitamin B_6, 100 mg.; inositol, 20 mg.

of water in an 18 × 150 mm. test tube with 0.1 ml. of the aqueous spore suspension. The inoculated medium was then incubated at 29° C. on a rotary shaker. After 16 hours, this inoculum was diluted 1 to 100 and used to inoculate the assays requiring vegetative cells (0.1 ml. per tube).

Assay Procedure. The compositions of the media used are shown in Table I. For the assays, 5.0 ml. of the desired medium was added to 18 × 150 mm. test tubes. The fraction or additive being tested was dissolved in deionized water to give concentrations that would cover the test range at a volume of 5.0 ml. or less and the pH adjusted to that of the medium. The test solution was added to each tube with deionized water to give a final volume of 10 ml. The tubes were capped with plastic caps and autoclaved at 122° C. for 4 minutes. After cooling, each tube was inoculated aseptically with the test organism.

The inoculated assays were incubated in a New Brunswick Psychrotherm incubator shaker (reciprocal action) at 285 r.p.m., with the tubes inclined at an angle of 50° from the horizontal. The spore assays used medium A and were incubated at 29° C. for 15 to 18 hours. Growth was determined with an Evelyn photoelectric colorimeter by measuring turbidity (absorbance) of the samples using a 720-mμ filter. Assays inoculated with the vegetative inoculum used medium B and were incubated at 35° C. In the latter cases, the absorbance was generally measured at 3, 4, and 5 hours on a Bausch and Lomb Spectronic 20 colorimeter at 720 mμ.

Ion Exchange Fractionation. The alfalfa juice extract prepared as described by Kohler and Graham (1951) and concentrated under vacuum to a thick sirup containing 640 grams of solids per liter was employed in the initial screening and subsequent fractionations. For fractionation, 2 liters of the concentrate were diluted with 2 liters of water. This solution could not be passed directly through the Dowex-50[H^+] column (Figure 1), since acidification of this

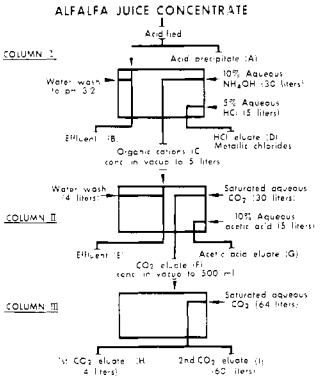

Figure 1. Flow sheet for separation procedures

material produced a flocculent precipitate which inhibited flow through the resin bed. The solution was acidified to pH 3 or lower using part of the cation exchange resin. The acid precipitate (*A*) was decanted from the resin, centrifuged, and washed with acidified water. The portion of the resin that was used for the acidification was added to the top of the resin bed (column I, Figure 1). The clarified supernatant was then passed through the resin bed and the procedure followed as shown in Figure 1. Fraction weights and resin volumes are given in Table II. In a larger scale preparation using the same procedure and starting with 20 liters of concentrate, 29.0 grams of the second CO_2 eluate (*I*) were obtained.

Countercurrent Distribution. The second CO_2 eluate (*I*), which amounted to 29 grams, was further purified by CCD in a robot-operated, 100-tube (200 ml. per tube) instrument employing water–acetic acid–2-propyl alcohol–*n*-butyl alcohol (60:7:10:30) as the solvent system. After 375 transfers, the run was divided into a series of fractions by arbitrarily combining consecutive tubes in groups of 25 or 50 (Table III) and assaying each fraction for its activity.

Table II. Data on Fractionation Scheme Described in Figure 1

Fraction	Weight, Grams	Column	Resin	Volume of Wet Resin, Liters
Alfalfa concentrate	1280	...	...	...
Acid precipitate (*A*)	180	...	...	...
Effluent (*B*)	781	I	Dowex-50[H^+]	6.0
Organic cations (*C*)	137	I	Dowex-50[H^+]	6.0
HCl eluate (*D*)	613	I	Dowex-50[H^+]	6.0
Effluent (*E*)	83	II	Dowex-1[OH^-]	2.0
CO_2 eluate (*F*)	36	II	Dowex-1[OH^-]	2.0
Acetic acid eluate (*G*)	23	II	Dowex-1[OH^-]	2.0
1st CO_2 eluate (*H*)	25	III	Dowex-1[HCO_3^-]	2.0
2nd CO_2 eluate (*I*)	7	III	Dowex-1[HCO_3^-]	2.0

Table III. Summary of the Countercurrent Distribution of the Second CO_2 Eluate (*I*)

Fraction	Tube No.	Weight, Grams	Major Compounds Present
1	0–50 OC[a]	0.44	.....
2	51–75 OC	0.85	.....
3	76–100 OC	2.85	Adenine, adenosine, hypoxanthine, guanosine, xanthine
4	101–125 OC	5.21	Guanine
5	126–150 OC	7.20	.....
6	151–175 OC	2.47	Inosine
7	176–200 OC	1.55	Isocytosine
8	201–225 OC	1.42	.....
9	226–275 OC	2.86	Cytidine
10	51–100 IC	5.06	.....
11	1–50 IC	0.33	.....

[a] OC is off the instrument, IC is tubes in the instrument.

Paper Chromatography. For microbiological assay, fraction 3, Table III, was chromatographed two dimensionally on 8 × 9.5 inch sheets of Whatman No. 3MM paper using a loading level of 0.5 mg. of 2-propyl alcohol; concentrated ammonium hydroxide (2 to 1) ascending was used for the first dimension and water ascending for the second dimension. Each two-dimensional chromatogram was divided into 16 sections, and the activity of each was determined in the manner described by Yacowitz *et al.* (1949). The location of spots which absorbed or fluoresced under short-wave ultraviolet light was compared with the active zones, and the compounds represented by these spots were cut out of the paper chromatogram and assayed separately.

For characterization and identification, a 1.25-mg. sample of the desired fraction was chromatographed on 18 × 18 inch sheets of Whatman No. 3MM paper employing the same solvent systems as above. Several of the compounds were not present in sufficient quantity in CCD fraction 3 for characterization. Therefore, each compound was isolated from the fraction in which it was most abundant. Absorbing spots corresponding to each of the nine compounds investigated (Figure 2) were cut out of the papers and combined with the comparable spots from other papers. The solutions were then taken to dryness in vacuo, dissolved in 10 ml. of ethanol, and ultraviolet spectra were run at pH 4.0, 7.0, and 8.0 for comparison with known compounds.

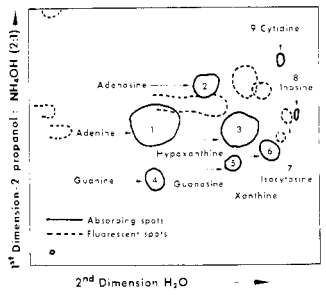

Figure 2. Two-dimensional paper chromatogram of isolated compounds

RESULTS AND DISCUSSION

The results of the initial experiments in this study verified the presence of factors in alfalfa, which are stimulatory to the growth of microorganisms. Of the bacteria tested, *B. subtilis* gave the best growth stimulatory response to the alfalfa extract in the paper disk assay. Therefore, further studies were conducted on this organism employing cystine medium fortified with brain heart infusion (1 to 1) again with good response. On medium A (Table I), *B. subtilis* spores gave a graded growth response to the alfalfa extract (Table IV) but showed essentially no response with soy or yeast extracts.

An attempt to evaluate the fractionation scheme encountered an activity pattern similar to that reported by Lee *et al.* (1961a), who found that the apparent activity of alfalfa extracts for *Neurospora sitophila* was governed by the presence of both inhibitory and stimulatory material. They further found that the balance, presence, or availability of these was also governed by the nature of the treatment of their extract. They, therefore, concluded that it would not be possible to estimate the recovery, or contribution, of the various fractions to the original activity. Similarly, the present authors have made no attempt to formulate a unit of activity for use in assessing the efficiency of the present separation scheme.

Fractionation on column I (Figure 1) was followed using a spore inoculum. None of the four fractions obtained from this column showed activity when assayed individually. The acid-insoluble precipitate (*A*) was inactive in all combinations. The effluent (*B*) in combination with either the organic cations (*C*) or the HCl eluate (*D*) was relatively inactive. The triple combination of effluent (*B*), organic cations (*C*), and HCl eluate (*D*), was about equal in activity to the original juice (Table IV). However,

Table IV. Graded Response of *B. Subtilis* Spores to Alfalfa Extract and Combined Fractions Thereof

Sample	Solids, Mg.	Absorbance
Medium A	...	0.000
+ alfalfa extract	5	0.036
+ alfalfa extract	10	0.108
+ alfalfa extract	15	0.252
+ alfalfa extract	20	0.432
+ alfalfa extract	25	0.553
Medium A + effluent (*B*) + organic cations (*C*) + HCl eluate (*D*)	Equivalent to 5	0.027
+ effluent (*B*) + organic cations (*C*) + HCl eluate (*D*)	10	0.076
+ effluent (*B*) + organic cations (*C*) + HCl eluate (*D*)	15	0.194
+ effluent (*B*) + organic cations (*C*) + HCl eluate (*D*)	20	0.398
+ effluent (*B*) + organic cations (*C*) + HCl eluate (*D*)	25	0.585

most of this activity could be accounted for by the double combination of the organic cations (*C*) and the HCl eluate (*D*). If a fraction from the ion exchange columns failed to show growth-stimulating activity by itself and in combination with other fractions, it was not subjected to further fractionation.

Since the HCl eluate was necessary to elicit the full response of the organic cation fraction, its composition was ascertained. The majority of the activity was not removed even after ashing to 1100° C., at which temperature 80% of the weight had volatilized. Apparently this factor(s) was inorganic. About half of the weight of the HCl eluate was ammonium chloride, whose presence primarily resulted from the eluants used for column I. It could be removed by ashing at 400° to 500° C. and accounted for a minor part of the activity of this fraction. Spectrometric analysis showed that the nonvolatile material consisted mostly of magnesium plus small amounts of several other elements. A combination of $MgCl_2$ plus KCl and $CaCl_2$ (based on analysis) accounted for most of the activity of the HCl eluate.

Based on these results, evidently assay medium A was deficient in certain nutrients essential for growth and/or germination of the test organism. The need for the HCl eluate was eliminated by optimizing the magnesium level. Decreasing the dextrose to 5 grams per liter and conducting the assay at pH 7.0 and 35° C. increased the rate of response to the basal medium several fold. All subsequent evaluations employed the medium with these modifications (medium B, Table I). A positive response to natural products—i.e., alfalfa, soy, and yeast extracts and brain heart infusion—was obtained on medium B employing either a spore or a vegetative inoculum (Table V). A vegetative inoculum was employed for the remaining fractionations as it eliminated the possibility of confusing a germinating factor with a growth-stimulating factor.

The suggestion that the alfalfa response might be caused by purines and pyrimidines followed from the similarity of its response to that obtained with natural products. Therefore, the fractionation scheme of Stark (1962) for the isolation of nitrogenous compounds from beet molasses was employed for the remaining fractionations. This scheme separates the purines and pyrimidines from the other nitrogen-containing materials. According to Stark (1962) the neutral substances, organic and inorganic acids, and pyrimidines such as uracil, uridine, and thymidine would pass directly through the Dowex 50(H^+) column and would be in the effluent (*B*). The organic cation fraction (*C*) would contain the amino acids, purines, and bases such as quaternary ammonium compounds. The more basic compounds passed through a Dowex-1 [OH^-] column (column II) into the effluent (*E*). The neutral amino acids and purines were eluted from column II with a saturated aqueous carbon dioxide solution giving the CO_2 eluate (*F*). The more acidic amino acids, such as glutamic and aspartic acid, were eluted with acetic acid (*G*). The major portion of the activity was concentrated in the CO_2 eluate (*F*) which contained 25% of the weight of solids added to the column. The neutral amino acids were separated from the purines on a Dowex-1 [HCO_3^-] column (column III). The neutral amino acids were eluted first, into the first CO_2 eluate (*H*), while the purines were eluted into the second CO_2 eluate (*I*). The latter fraction contained most of the activity and only 10% of the weight. The second CO_2 eluate was further purified by CCD and divided into 11 more fractions. The most active fraction (fraction 3, Table III) gave a further substantial increase in concentration of the activity. However, some activity was also found in several of the other fractions.

Final purification of the growth stimulating factors was accomplished by paper chromatography. The major spots were identified by a paper chromatographic comparison with known compounds and by ultraviolet spectral studies. The identified compounds are shown in Figure 2. They are adenine, adenosine, hypoxanthine, guanosine, xanthine, guanine, inosine, isocytosine, and cytidine. The CCD fractions from which these were isolated are given in Table III. There was some overlapping of compounds in the various fractions. Only adenine has been previously reported present in alfalfa (Vickery and Leavenworth, 1925).

The relative amounts of adenine, adenosine, hypoxanthine, and guanosine present in the second CO_2 eluate (*I*) was determined spectrophotometrically. Hypoxanthine was present in such minute amounts that its concentration could not be determined in the organic cation fraction or the original alfalfa juice. However, the other three were assayed and the results are summarized in Table VI. A combination of adenine, adenosine, hypoxanthine, and guanosine at the levels present in the second CO_2 eluate (*I*) can account for all of the growth-stimulating activity

Table V. Growth-Stimulating Effect of Natural Products

	Absorbance				
	3.0 hr.	3.5 hr.	4.0 hr.	4.5 hr.	5.0 hr.
Spore Inoculum					
Medium B only	...	0.004	0.009	0.032	0.097
+ Alfalfa[a]	...	0.086	0.229	0.495	...
+ Yeast extract	...	0.131	0.319	0.638	...
+ Soy extract	...	0.051	0.149	0.357	...
+ Brain heart infusion	...	0.086	0.229	0.538	...
Vegetative Inoculum					
Medium B only	0.013	0.046	0.155	...	...
+ Alfalfa	0.051	0.174	0.420	...	...
+ Yeast extract	0.119	0.301	0.602	...	...
+ Soy extract	0.041	0.137	0.357	...	...
+ Brain heart infusion	0.066	0.187	0.409	...	...

[a] Levels used for all samples, 2 mg. per tube.

Table VI. Purine and Nucleoside Composition

Fraction	Adenine, %	Adenosine, %	Guanosine, %	Hypoxanthine, %
Alfalfa juice	0.17	0.25	0.36	...
Cations (*B*)	0.64	1.08	2.91	...
CO_2 eluate (*I*)	14.50	14.48	13.10	1.89

of that fraction. The combination of the three most abundant compounds, adenine, adenosine, and guanosine, at the levels present in the original extract can account for almost all the activity of this extract (Figure 3). At concentrations ranging from 0.2 to 250 μg., all of the purines and pyrimidines isolated stimulated the rate of growth of the organism. Adenine, hypoxanthine, and inosine produced the greatest and the most consistent stimulatory responses. Dosage-response curves for these three compounds are presented in Figure 4. Variable responses were caused by the other isolated compounds, and graded responses usually were not obtained. Variable responses to these types of compounds by microorganisms have been previously observed (Weinman *et al.*, 1964; Lovtrup and Shugar, 1961; Jeener and Jeener, 1952).

The growth-stimulating properties of purines, pyrimidines, and their nucleosides on microorganisms has been previously observed. Several reviews of the literature on this subject prior to 1945 are available (Loring, 1944; Knight, 1945). More recently, Brewer *et al.* (1946) reported uracil, adenine, and guanine stimulated growth and spore production of *Bacillus anthracis*. Studies on

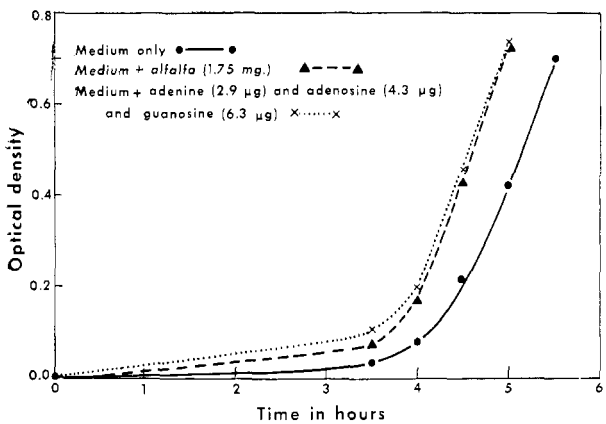

Figure 3. Growth stimulatory response of *Bacillus subtilis* to alfalfa and to the combination of adenine + adenosine + guanosine at comparable concentrations

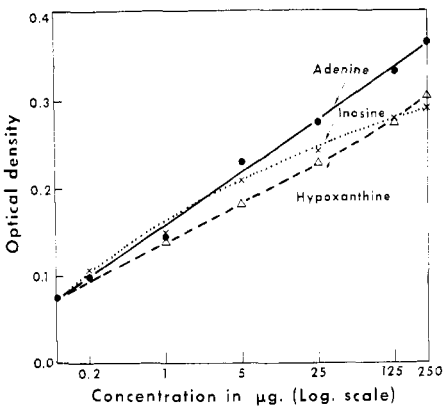

Figure 4. Dosage-response curves for adenine, inosine, and hypoxanthine on medium B

growth stimulants in pancreas extracts for *Streptococcus lactis* showed that inosine, hypoxanthine, and adenine were the active components (Koburger *et al.*, 1963). Williams and co-workers have extensively studied unidentified growth factors for *Lactobacillus bulgaricus* Georgia strain. In connections with these studies they found (Weinman *et al.*, 1964) that adenine, RNA, alkaline digests of RNA and Mg^{2+} consistently stimulated the growth of the organism. Several other purines, pyrimidines, and related compounds occasionally stimulate growth responses by the organism.

Demain (1958), in the course of development of a minimal medium for quantitative studies with *B. subtilis* (ATCC 6051), found that a mixture of vitamins, purines, and pyrimidines failed to increase colony formation but gave no data on the effect of these compounds on the production of turbidity in liquid culture as the criterion of growth. Feeney *et al.* (1948) reported a study dealing with the general nutritional factors conducive to rapid growth and formation of relatively high levels of subtilin by *B. subtilis* (ATCC 6633), the same strain being employed in this investigation. They found that supplementation with a mixture of growth factors, including adenine, guanine, uracil, and xanthine gave varying results ranging from no apparent effect to moderate stimulation. The only other reports in the literature of purines and nucleoside requirements for *Bacillus subtilis* were for mutant strains (Guthrie and Lu, 1964; Demain, 1964).

Various *B. subtilis* strains have been shown to produce adenosine (Koaze *et al.*, 1962; Aoki *et al.*, 1962), hypoxanthine (Hara *et al.*, 1964), inosine (Aoki *et al.*, 1963; Shiro *et al.*, 1964b), and guanosine (Shiro *et al.*, 1964a). Lewis *et al.* (1953), looking for growth-stimulating supplements for chicks, reported that a dried acid precipitate from whole cultures of *B. subtilis* (ATCC 6633) gave a good chick response. Lillie *et al.* (1953) showed that dried *B. subtilis* cells produced a growth response in chicks. Since dried *B. subtilis* fermentation solubles have also been shown to be good sources of the alfalfa-whey factor for chicks (Rasmussen, 1965), it is possible that the purines and nucleosides that are present in these culture preparations could be the active constituents. In this connection, Barnett *et al.* (1956), studying the response of chicks to a penicillin fermentation residue, showed that adenosine isolated from the residue produced a positive response. However, Ritchey *et al.* (1956) and Dam *et al.* (1961) were not able to substantiate Barnett's findings. There are not many reports in the literature of these types of compounds being tested with chicks or other animals. Apparently, this is an area which needs to be further investigated.

ACKNOWLEDGMENT

The authors express their appreciation to K. V. Smith and J. W. Nelson for their technical assistance in the preparation of the alfalfa juice concentrate; to A. L. Potter for the spectrophotometric analysis; to M. Hooper and K. Jensen for laboratory assistance; and to M. Burger of Wisconsin Alumni Research Foundation for development of the assay procedure and helpful discussions throughout the course of the work.

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Received for review September 5, 1967. Accepted December 4, 1967. 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.