FEED SUPPLEMENTS

Genus *Bacillus* as a Source of Growth Stimulants

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A search for antibiotic feed supplements has been centered on the genus *Bacillus* because many strains of bacteria of this group grow very rapidly on simple media to produce a wide variety of antibiotics. The empirical approach, involving chick-feeding tests at an early stage of selection, has yielded several stocks which give 5 to 10% greater growth responses than the control chicks at 10 weeks. More intensive investigations of these stocks, now in progress, are intended to identify the growth-promoting substances, to provide methods for the rapid assay of these substances, and to develop economical forms of the supplements.

INVESTIGATIONS OF FEED SUPPLEMENTS capable of stimulating chick growth continue on an extensive scale. New nutritional factors are attributed to fish solubles, whey, grass juice, and various microbial products. As so much is already known about chick nutrition, this activity may seem surprising. Work with chicks has helped, however, in the discovery and development of many vitamins and growth factors, and microbiological and synthetic processes have been developed to meet the requirements of the poultry feed industry for these new supplements. The poultry industry has supplied the major incentive also for the recent development of nutritional use of antibiotics. Penicillin, chlorotetracycline (Aureomycin), oxytetracycline (Terramycin), and bacitracin are all used commercially in feed supplements. The equivalent of 258,000 pounds of pure antibiotics was used in 1952, according to the U. S. Tariff Commission; the wholesale value has been estimated to be in the neighborhood of $20,000,000 annually.

When we reflect on the remarkable improvement during recent years in the efficiency with which feed becomes bird, it is not surprising that this profitable field of research should be a scene of booming activity. Up to 4 weeks of the chick's age, a pound of bird can be obtained with 2.25 pounds of feed; at 10 weeks each pound of bird may represent only 2.75 pounds of feed. Shortly before World War II, poultrymen were able to produce with only about half that efficiency. Whereas the value of vitamin and amino acid supplements has been the avoidance of limiting deficiencies of essential nutrients, the basis of the marked beneficial effect of antibiotics on efficiency of feeds is not clearly explained, but the gains may be caused in part by reduction of burden of maintenance of intestinal microorganisms which, on modern rations, may serve no compensating purpose.

The prior evidence for unidentified growth factors in various microbial products (discussed below) together with the hope of finding more economical antibiotic feed supplements, led the authors about 2 years ago to initiate a search for microorganisms capable of promoting the growth of chicks. Attention was centered upon bacteria of the genus *Bacillus*, not only because these bacteria produce a large number of antibiotics that have not been investigated as intensively as those from *Streptomyces*, but also because many strains of *Bacillus* grow with exceptional rapidity upon simple media (*3, 7*). The propagation characteristics appear to be particularly adaptable to inexpensive equipment and to continuous propagation.

Screening Program

The screening program that was envisaged presented certain statistical problems because of the low magnitude of the responses expected, and the relatively high variability inherent in chick-feeding tests. On the other hand, it was expected that hundreds, if not thousands, of different bacterial strains would have to be fed if the chances of finding useful strains were to be reasonably high. As a matter of efficiency, the procedure of refeeding only stocks that gave the largest and most reproducible chick growth responses was adopted. Some good strains may well have escaped detection; on the other hand, certain stocks would be expected by chance alone to give a run of good feeding tests. However, it was thought more efficient to defer the elimination of such stocks until intensive work on fractionation was undertaken.

Selection of Antibiotic *Bacillus* Strains

The first step of the screening program involved the solicitation of antibiotic strains of *Bacillus* or their isolation from soil plates. All of those solicited stocks which produce named antibiotics (with the exception of two subtilin-producing strains), either failed to grow or failed to produce antibiotics under submerged aeration on a simple beet molasses medium when they were first tested, or else they subsequently failed to stimulate chick growth. A large number of strains of antibiotic *Bacillus* selected in the Fermentation Division of the Northern Regional Research Laboratory, Peoria, Ill., were made available through the courtesy of R. W. Jackson and R. G. Benedict. Several of these showed some promise and one (*B. subtilis* NRRL B-1474) is discussed below. Of the total of approximately 2100 stocks grown in shake flasks, however, all but approximately 100 were isolated from soil plates either by the "crowded plate" technique or by the technique described by Kelner (*5*) with *B. megaterium*, *Micrococcus flavus*, *Escherichia coli*, and *Xanthomonas phaseoli* as test bacteria or by an anaerobic

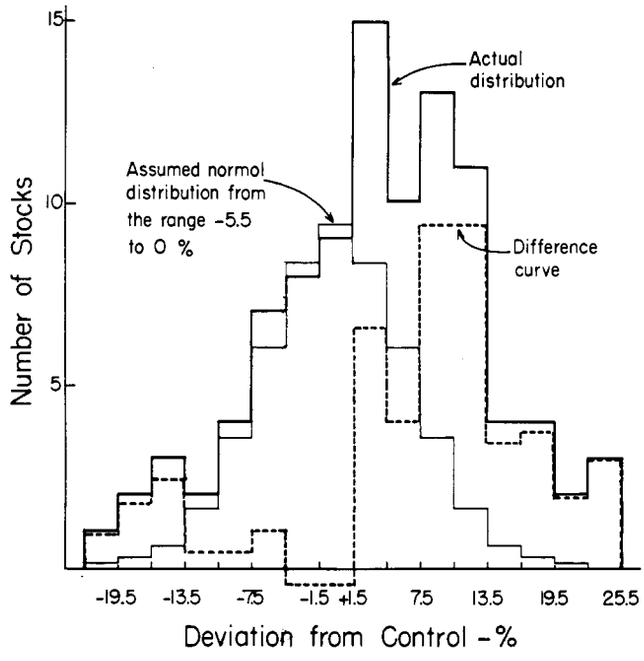

Figure 1. Frequency distribution for chick growth – promoting effects of *Bacillus* stocks

modification (*9*) with a *Clostridium* sp. One of those selected from crowded plates (*B. subtilis* NRRL B-1466) and one isolated against *B. megaterium* (*B. subtilis* var. *aterrimus* NRRL B-1471) are discussed below.

The more promising strains have been deposited in the Stock Culture Section of the Northern Regional Research Laboratory. The authors are indebted to W. C. Haynes and R. W. Kuehne of this organization for taxonomic identifications.

Incubation, Propagation, Feeding Tests As the second step of the screening program, all the stocks were incubated on beet molasses medium (*7*) in shake flasks. Those that grew well were assayed by serial dilution, agar streak, or other methods against the test bacteria. The more active stocks were selected for propagation on a 10-liter scale in propagators capable of excellent air dispersion. These propagations, which constituted the third step of the screening procedure, have been described (*7*). The propagations were characterized by cell yields (dry weight) of 20 to 60% of the sugar supplied and growth periods of 6 to 10 hours. The whole cultures routinely were drum-dried under atmospheric pressure after vacuum concentration, although in a few cases vacuum drying was used or the sirups were fed directly. The losses of antibiotic activity were greater on drum-drying than by the other procedures, but it was thought that this might be an advantage because the more stabile antibiotics would tend to be selected. Various drying aids were tested; paper pulp proved most effective. When used it comprised about 10% of the weight of the product. The products that were more active antibiotically were selected for chick feeding tests in the fourth step of the screening.

The greater part of the chick tests have been conducted under contract with the Wisconsin Alumni Research Foundation. The basal rations were a corn and soybean oil-meal all-vegetable-protein diet well supplemented with minerals and vitamins, including 30 p.p.m. of crystalline cyanocobalamin (vitamin B_{12}), and the same ration supplemented with 3% of fish meal. The former was used in only a few early tests. Commercial-hatchery New Hampshire chicks were grouped 20 to a pen (equal numbers of each sex) and fed *ad libitum*. Procaine penicillin G at 10 p.p.m. was fed as a positive control in most tests. The penicillin invariably promoted growth, the response varying from 12 to 35% (average 21%) at 4 weeks; and from 6 to 21% (average 10%) at 10 weeks. The standard deviations of the increased growth due to penicillin were 6.3 and 5.4% for 4 and 10 weeks.

All tests were run for 4 weeks and those that appeared especially promising were continued for 10 weeks. Ninety-seven different bacterial stocks were fed for the first time in the Wisconsin tests. The 4-week data from such cultures are not subject to bias through selection, and so it is of interest to consider the frequency distribution of responses (Figure 1). If a normal distribution, centered on zero response, is subtracted from the actual distribution to account for that proportion of the total cultures fed which are without real effect on the growth of the chicks, the difference curve indicates that only a small proportion of the cultures inhibit growth and that a large proportion of the cultures stimulate chick growth. Most of these cultures were fed at 0.1 to 0.3% of the ration.

A further considerable number of crude whole cultures were fed initially by the Ray Ewing Co., Pasadena, Calif. A number of cultures considered worthy of further investigation were selected from these tests for repeated feeding in the same manner as those selected from tests by the Wisconsin Alumni Research Foundation.

Selection of Best Cultures The selection of the best cultures from the standpoint of realness of the response, magnitude, and reliability formed the next stage of the screening. Approximately 56 stocks were fed at least twice, and 30 of the more promising of these were fed three or more times. To provide material, the more promising stocks were regrown first on a 10-liter scale and then on a 150-liter scale in this laboratory, or on a 15,000-liter scale through the generous aid of J. M. Sudarsky of the Pacific Yeast Products Co., Wasco, Calif. (*7*). Concurrently, various stocks were dropped for a variety of reasons: failure to grow reliably, failure to produce a high level of antibiotic activity routinely, failure to promote growth of chicks through 10 weeks reliably, failure to maintain activity on drum drying, and duplication of previously tested cultures selected independently. Antibiotic patterns were used primarily for recognizing duplicated strains. Paper chromatography with bioautographic detection proved very useful (*13*), particularly in combination with simple fractionation procedures.

Fractionation Of Cultures The sixth stage of the investigation (now in progress) involves fractionation of the crude cultures for the purpose of isolating and identifying the active factors. This is necessary to the development of more rapid assay methods than the chick test for control of production of the active factors, both in potential use and in research directed at raising yields of the active factors. Fractionation results may also prove useful in the economical preparation of concentrates by avoiding the necessity for drying the whole culture. It has been found, for example, that acidification of whole culture (or cell-free liquid in many cultures) precipitates some antibiotics near-quantitatively. These may then be recovered economically in a yeast centrifuge. This behavior is similar to that for tyrothricin and subtilin, although the antibiotics are clearly different.

Considerable attention has been paid to the subtilin-producing strain of *B. subtilis* (ATCC 6633). Strain NRRL B-1467 of *B. subtilis* was selected independently on the basis of chick-growth stimulation and subsequently was shown to produce subtilin in similar yields, as judged by isolation. Some typical results with these two strains and with partially purified subtilin are

shown in Table I. In addition to the two tests shown, positive responses to crude products were obtained in at least seven other tests at various laboratories, questionably positive tests were obtained in two other tests, and an apparent depression of growth was observed only once. However, because of the bias inherent in the procedure, the evidence in favor of a real stimulation may not be so good as it appears to be. A substantial response to subtilin (50 p.p.m.) was reported by Heuser and Norris (*4*). A number of tests with lower levels of subtilin, on the order of 5 to 20 p.p.m., have failed to give consistent stimulations.

An anomaly is presented by the fact that the antibiotic activity of subtilin is destroyed by digestive enzymes, so that it must be questioned whether the chick growth-promoting activity resides in the antibiotic activity of subtilin. Furthermore, it proved impossible to assay or to isolate reasonable amounts of subtilin or material with similar solubility properties from a drum-dried acid precipitate of whole culture of ATCC 6633 which had given good chick-growth responses. Thus it is uncertain whether the response is due to subtilin, to a degradation product of subtilin, or to some other constituent of the culture.

Although considerable work has been done with respect to the nutritional and propagation conditions required for subtilin production by ATCC 6633, no attempt to increase yield by strain selection has been reported as yet. If the chick growth stimulation appears important from the qualitative standpoint, it appears likely that strain selection could give economically practical supplements.

Another strain of *B. subtilis*, NRRL B-1474, has given relatively reproducible feeding results, as is shown in Table II. The growth response has been obtained consistently with relatively high levels, in the range of 1 to 4% of the ration. Lower levels and particularly the question of a response in the presence of penicillin have not been tested adequately. The strain produces a complex mixture of antibiotics, four of which have been separated from one another. None appears to be identical with any of the previously reported antibiotics from *Bacillus*. It cannot yet be said whether the chick growth-promoting activity of this strain is related to any of the antibiotics produced by it.

A third strain of *B. subtilis*, NRRL B-1466, has received considerable attention because it gave a number of good growth responses when fed at approximately 0.1% of the ration as dried whole culture. However, in other tests 0.25 to 1.0% proved optimal and some lots gave no effect. Variably severe heat processing may account for the lack of response from certain lots. A new antibiotic was obtained from this strain, but concentrates of it failed to stimulate chick growth.

A strain of *B. subtilis* var. *aterrimus*, NRRL B-1471, has given fairly consistent growth stimulations at low levels. The growth stimulation is given by a concentrate of one of two antibiotics produced by this strain. Butanol extraction of an acid precipitate of the culture filtrate gives one of the antibiotics and the chick growth-promoting activity. Chick-growth results are shown in Table III.

The butanol extraction gave an approximate 40-fold concentration of the desired antibiotic (solids basis). The specific potency has been increased to a total of approximately 6000-fold over the original culture solids by a series of solvent extractions including countercurrent distribution. It is active in the 1 to 10 p.p.m. range for a number of Gram-positive bacteria, including some strains of *Clostridium*. Chick feeding tests have not yet been made with the most highly purified material, but if the extrapolation from the butanol extract proves to be valid, the antibiotic would be expected to stimulate chick growth with a concentration in the order of 1 gram per ton of ration—a level somewhat less than that required with procaine penicillin G.

This antibiotic has been named "aterrimin" after the varietal name of the producing bacterium.

Evaluation of Products

The final stage of the program is evaluation. As relatively little can be said regarding the value of the products, this report is very much in the nature of a progress report. It is appropriate, however, to express appreciation to various researchers who have already been able to initiate and, in some cases, to complete preliminary feeding tests. The independent feeding tests are highly valuable in extending knowledge of the reliability of the responses under varied conditions; and they may also help to indicate whether commercially useful products might be developed from these bacteria. With such considerations in mind, the authors are prepared to distribute stock cultures of the more promising strains on request.

It is desirable to indicate the part that these materials might play in the feed-supplement field. Because of the very low price at which penicillin now sells in the feed supplement trade, it is apparent that the specific potency of crude cultures would have to be increased many fold before the products could compete simply as alternates to the present feed antibiotics. A matter of greater interest is the question whether these materials would give significant responses in combination with the commercial antibiotics. No definite answer can be given as yet to this very important question. Likewise, the possibilities that any of these materials may supply the unidentified factor of fish solubles remains unproved, although some promising indications have been reported by Bird (*8*).

The use of microbiological materials as feed supplements has been outstandingly important. Much of the early work on vitamins of the B complex involved feeding tests with yeast, a

Table I. Chick-Growth Response with Subtilin and with *B. subtilis* ATCC 6633 and NRRL B-1467

		Weight or % of Control		Feed per Gram of Gain or % of Control	
Test[a]	Basal Diet and Supplements	At 4 weeks	At 10 weeks	At 4 weeks	At 10 weeks
2/53	Vegetable protein with 3% fish meal, g.	263	1238	2.49	3.08
	Procaine penicillin G, 10 p.p.m., %	124	107	79	83
	ATCC 6633, drum-dried acid precipitate of whole culture, %				
	3 g. per 100 g.	115	107	87	86
	1 g. per 100 g.	117	108	87	83
	0.3 g. per 100 g.	113	102	90	91
	Subtilin (70%), 300 p.p.m., %	121	103	84	86
7/51	Vegetable protein, g.	245	1205	..	2.54
	Chlorotetracycline,[b] 10 p.p.m., %	116	105	..	96
	Bacitracin, 10 p.p.m., %	111	106	..	94
	B-1467, drum-dried whole culture, %				
	0.07 g. per 100 g.	106	107	..	104
	0.14 g. per 100 g.	109	105	..	101
		At 6 weeks			
Heuser and Norris(*4*)	Vegetable protein, g.	564			
	Subtilin, 50 p.p.m., %	106			
	6 other antibiotics, 50 p.p.m., %	107 to 113			
	Animal protein, g.	599			
	Subtilin, 50 p.p.m., %	116			
	6 other antibiotics, 50 p.p.m., %	110 to 113			

[a] Dated tests conducted by Wisconsin Alumni Research Foundation.
[b] Aureomycin.

Table II. Chick-Growth Response with *B. subtilis* NRRL B-1474

Test[a]	Basal Diet and Supplements	Weight or % of Control: At 4 weeks	Weight or % of Control: At 9 weeks	Feed per Gram of Gain or % of Control: At 4 weeks	Feed per Gram of Gain or % of Control: At 10 weeks
3/51	Vegetable protein with 2.5% meat scraps, g.	300	1197	..	..
	Aurofac, Baciferm, and TM-5,[b] %	106 to 111	98 to 102	..	..
	B-1474, drum-dried whole culture, %				
	0.25 g. per 100 g.	102	102	..	..
	0.5 g. per 100 g.	106	100	..	..
	1 g. per 100 g.	113	108	..	..
	2 g. per 100 g.	108	106	..	..
	4 g. per 100 g.	128	110	..	..
		At 10 weeks			
7/51	Vegetable protein, g.	245	1205	..	2.54
	Chlorotetracycline,[c] 10 p.p.m., %	116	105	..	96
	Bacitracin, 10 p.p.m., %	111	106	..	94
	B-1474, drum-dried whole culture, %				
	1.5 g. per 100 g.	109	105	..	99
	4 g. per 100 g.	115	109	..	92
11/52	Vegetable protein with 3% fish meal, g.	258	1103	2.67	2.61
	Procaine penicillin G, 10 p.p.m., %	122	117	85	90
	B-1474, drum-dried unwashed cells, %				
	3 g. per 100 g.	112	113	96	100
2/53	Vegetable protein with 3% fish meal, g.	263	1238	2.49	3.08
	Procaine penicillin G, 10 p.p.m., %	124	107	79	83
	B-1474, drum-dried whole culture, %				
	3 g. per 100 g.	117	105	83	75
	3 plus penicillin (10 p.p.m.)	128	110	79	76

[a] Test dated 3/51 conducted by Ray Ewing Co., others by Wisconsin Alumni Research Foundation.
[b] Commercial feed supplements containing chlorotetracycline (Aureomycin), bacitracin, and oxytetracycline (terramycin), respectively, to give 9 to 10 g. per ton of ration.
[c] Aureomycin.

material that has entered indirectly into the diets of early man in many parts of the world. Recovery of by-product yeast from breweries and distilleries has been a very important development of the past two decades. The use of a microorganism for the primary production of a single important feed ingredient appears to have begun with the use of fungi of the genera *Ashbya* and *Eremothecium* for the production of riboflavin. This development dates from approximately 15 years ago and followed the earlier process of recovery of riboflavin concentrates from the fermentation by-products in the production of acetone and butanol by *Clostridium acetobutylicum*. The mode of attack on the development of cobalamin-containing feed supplements by microbial processes thus quickly became apparent to many investigators. Astonishingly rapid progress has been made with this problem and it appears not to have tapered off yet.

More or less coincidental with the cobalamin development has been the development of feed supplements of chlorotetracycline (Aureomycin), penicillin, oxytetracycline (terramycin), and bacitracin, although it has involved only an adaptation of antibiotics already developed for medical uses.

Future Developments

The question may be asked; What further developments lie in the near future for microbiological feed supplements? The situation appears auspicious. The principal stumbling block in the development of microbiological processes for the factors in fish solubles (*2*), whey (*10*), and green vegetable juices (*6*) is the lack of rapid assay procedures. A rapid assay procedure can substitute for a tremendous amount of inspired guesswork and technical skill. A second qualification is that what appear to be new factors sometimes come to be explained in terms of known nutritional facts. An example is the recent report (*12*) that the factor in brewer's yeast that prevents the hock disorder in turkey poults actually is niacin plus an antioxidant that stabilizes vitamin E.

Very interesting results have been reported (*1, 11*) regarding stimulation of the growth of chicks in the presence of penicillin by feeding viable coliform bacteria (*Aerobacter aerogenes* or *Escherichia coli*). Romoser *et al.* (*11*) obtained positive results on both soybean oil–meal and fish-meal rations with as little as 0.003% of lyophilized bacterial cells. Such a response would be most intriguing from the commercial standpoint if the technical problems involved in the distribution and use of a viable supplement could be solved. A similar problem in the development of active dry yeast for baker's use has been solved reasonably well.

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Table III. Chick-Growth Response with *B. subtilis* var. *aterrimus* NRRL B-1471

Test[a]	Basal Diet and Supplements	Weight or % of Control: At 4 weeks	Weight or % of Control: At 10 weeks	Feed per Gram of Gain or % of Control: At 4 weeks	Feed per Gram of Gain or % of Control: At 10 weeks
9/52	Vegetable protein with 3% fish meal, g.	256	1137	2.10	2.67
	Procaine penicillin G, 10 p.p.m., %	117	106	86	94
	B-1471, vacuum-dried whole culture, %				
	0.025 g. per 100 g.	109	107	96	93
	0.075 g. per 100 g.	111	110	95	91
1/53	Vegetable protein with 3% fish meal, g.	271	1168	2.80	3.43
	Procaine penicillin G, 10 p.p.m., %	122	107	73	78
	B-1471, %				
	Dried residue from butanol extraction, 0.4 g. per 100 g.[b]	111	106	86	88
	Butanol extractables				
	22 p.p.m., dry basis	111	104	86	89
	67 p.p.m., dry basis	122	105	73	89
4/53	Vegetable protein with 3% fish meal, g.	255	1325	2.47	2.74
	Procaine penicillin G, 10 p.p.m., %	122	105	80	90
	B-1471, %				
	Dried residue from butanol extraction, 0.8 g. per 100 g.	107	103	93	91
	Butanol extractables				
	46 p.p.m., dry basis	109	103	97	94
	140 p.p.m., dry basis	107	105	92	97

[a] All tests conducted at Wisconsin Alumni Research Foundation.
[b] Lower levels less effective.

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CAROTENE

Determination in Alfalfa Meal Treated with *N,N'*-Diphenyl-*p*-phenylenediamine

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The AOAC method for determination of carotene in dehydrated alfalfa meal is not suitable for use with alfalfa meal treated with *N,N'*-diphenyl-*p*-phenylenediamine, as the passage of the diamine through the magnesium oxide adsorbent results in the production of a yellow color. A method is presented which employs tricalcium phosphate as the adsorbent, and which can be used when the diamine is present. By means of this method the potency of *N,N'*-diphenyl-*p*-phenylenediamine as a carotene stabilizer was evaluated.

BEAUCHENE *et al.* (*1*) have shown that the presence of *N,N'*-diphenyl-*p*-phenylenediamine on alfalfa meal interferes with the determination of carotene when the AOAC method of analysis is used. The magnesia of the adsorbent caused alteration of the diamine in some manner, producing a yellow color which contaminated the carotene fraction and caused abnormal carotene values.

N,N'-diphenyl-*p*-phenylenediamine has been approved by the U. S. Department of Agriculture for use on alfalfa meal as a carotene stabilizer (*4*). In view of the results of Beauchene *et al.*, it is probable that this chemical is less efficient as an antioxidant than was indicated by previous work (*2, 3, 8*). It is of interest, therefore, to have available a method that will accurately evaluate the degree of carotene preservation in alfalfa meal treated with this substance. Furthermore, because diamine-treated meal may be moving in commercial channels and thus would be subject to quality control, a method for carotene determination is needed which will not be influenced by the presence of the antioxidant. This investigation was initiated for the purpose of developing such a method.

Experimental Work

The specific problem involved is the selection of an adsorbent that will not react with the diamine to produce a color. At the same time, the adsorbent must separate the carotene adequately from the noncarotene pigments of the meal.

Mitchell, Schrenk, and Silker (*6*) reported that powdered tricalcium phosphate would adsorb chlorophyll and xanthophylls from a Skellysolve B solution, but that carotene was adsorbed weakly and could be removed easily from the adsorbent by washing with Skellysolve B. This adsorbent was investigated as a substitute for magnesium oxide in the AOAC method of analysis. By adding tricalcium phosphate to a solution of the diamine in Skellysolve B and shaking the mixture vigorously for a few minutes, it was determined that it did not react with the diamine to produce a color.

The quantitative aspects of the proposed adsorbent were studied by analyzing untreated alfalfa meal samples for carotene by the AOAC method and by a modification of the AOAC method which permitted the use of tricalcium phosphate as the adsorbent. The modification consisted of transferring the extract obtained by the AOAC extraction procedure to a 600-ml. beaker and diluting with Skellysolve B to about 300 ml. The extract was concentrated to 30 to 40 ml. on a steam plate to drive off most of the acetone. This was the procedure used by Wall and Kelley (*10*) for eliminating acetone from the extract, and is necessary to prevent elution of noncarotene pigments by the acetone during adsorption. The concentrated extract was drawn through a 9-cm. column of a 1 to 1 mixture by weight of powdered tricalcium phosphate and Super Cel. The column was washed with Skellysolve B until the eluate was colorless. Less than 100 ml. of Skellysolve B was required to accomplish this. The eluate was diluted to 250 ml. with Skellysolve B and color intensity was measured at 4360 A. with a Beckman DU spectrophotometer.

Carotene values obtained by the two

Table I. Carotene Content of Untreated Alfalfa Meals

(As obtained by AOAC method and by a modification which permitted use of tricalcium phosphate as adsorbent)

Sample	AOAC Method, Mg./100 Grams	Calcium Phosphate Method, Mg./100 Grams
1	21.9	20.7
2	22.3	21.8
3	18.4	17.5
4	21.6	21.0
5	21.8	20.4
6	16.8	16.2
7	12.9	12.5
8	11.6	11.0
9	6.5	5.8