Table II. Stability of Color at 25° C.

Hours after First Reading	Absorbance Loss, % In dark	Absorbance Loss, % In artificial light
4	0.4	0.9
21	1.5	5.6
28	2.1	8.5
52	3.2	16.0

Table III. Average Milligrams of 2-Ethyl-1,3-hexanediol Recovered from Various Surfaces

Surface	Applied	Recovered
Glass plates	241	242
Unextracted cloth	248	249
Extracted cloth	248	245
Human skin	477	465

Figure 4 shows that a reaction time of 60 minutes is required to develop maximum absorbance. The absorbances failed to check well when duplicate samples were heated for shorter periods.

The data presented in Table II show that the color fades after development at the rates of 0.1% and about 0.25% per hour in the dark and in artificial light, respectively. Therefore, error due to fading is insignificant.

The results of the analyses for 2-ethyl-1,3-hexanediol recovered from glass, cloth, and human skin are shown in Table III. Quantitative recoveries were obtained from glass and cloth. The recovery from skin was 97.5%. Failure to obtain quantitative recovery from skin may have been due to absorption, a phenomenon which is being investigated. The absorbance due to material washed from an untreated arm varies somewhat with individuals and usually is less than 0.010.

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FORAGE NITRATE

Improved Microbiological Method for Nitrate Determination

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Nitrate is quantitatively reduced to nitrite by nitrate reductase, an enzyme produced by certain bacteria. *P. oleovorans* is ideal for such use, because this enzyme is always present, although the organism does not have a nutritive requirement for nitrate. Nitrite reductase, an enzyme which frequently interferes with this method, is not formed. Prior isolation of nitrate is not required and the usual biological tissues and fluids contain no apparent inhibitor for this enzyme. The method as described covers 2 to 200 γ, in nitrate content at any one sample dilution.

Consumption of forages and silages containing as little as 1.5% potassium nitrate, on a dry basis, frequently causes death of sheep and cattle (*5*). Any rapid method for the determination of nitrate capable of giving dependable results on a variety of materials, without requiring numerous modifications or separation procedures, would be of great value. The numerous simple chemical or colorimetric methods adaptable to relatively pure systems are usually not applicable to complex mixtures such as plant or animal tissues, the recoveries of added nitrate differing greatly depending on the particular method and plant species involved (*6, 7*).

The use of a partially purified adaptive enzyme, nitrate reductase, as suggested by Nason and Evans (*8*) had little to offer, because the isolation of the enzyme, which was rather unstable, required considerable time. Garner and coworkers (*4*) contributed greatly to the usefulness of the enzymatic method by demonstrating that this enzyme which reduces nitrate to nitrite did not require isolation, prior to use, but could be produced by growing a suitable microorganism directly in the test tube containing the solution to be investigated. These authors employed an unidentified bacillus obtained from sheep rumen to produce the desired enzyme. The nitrite formed was then determined colorimetrically. This microbiological method, although accurate and precise, entails considerable preparation. The stock culture must be subcultured on nitrate-containing agar, but excess subculturing on such a medium allows the formation of an undesirable adaptive enzyme, nitrite reductase. After culturing, the organism must be washed repeatedly to remove all nitrate and nitrite. In addition to the possible presence of nitrite reductase, the time of incubation of the nitrate solution with the bacteria is very critical, the greatest effect being noticed at low nitrate concentrations.

Pseudomonas oleovorans (Lee and Chandler) (American Type Culture Catalog)

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ATCC No. 8062 has important advantages over the organism employed by Garner and coworkers for the quantitative determination of nitrate. The present paper describes a procedure for using this organism.

Materials and Methods

Chemical Procedure. For purposes of comparison a chemical method was employed which is a modification of the procedures of Davisson (*2*) and Strowd (*9*) as made by Whitehead (*10*). This method, a modified Devarda procedure in which the nitrate is reduced to ammonia by Devarda's alloy in an alkaline medium, has given good reproducibility for most forages. However, it is of little value in the presence of urea, and the distillations require considerable attention, as there is a great tendency to foaming. After hot water extraction of a 5-gram sample and precipitation of any protein by addition of 5 ml. of a saturated neutral lead acetate solution, a suitable aliquot (200 ml.) of supernatant is placed in a Kjeldahl flask to which are added 25 ml. of a 5% sodium hydroxide solution. The total volume is adjusted to about 300 ml. Any ammonia nitrogen present is removed by distilling off about 150 ml. After cooling, the volume is again adjusted to 300 ml. and 3 to 5 grams of Devarda's alloy are added to reduce any nitrate or nitrite to ammonia, which is then determined by titration after distillation in the usual Kjeldahl manner.

Culture of the Microorganism. *Pseudomonas oleovorans*, ATCC No. 8062, was used in all experiments reported in this study, although many cultures of this species isolated from soluble oil emulsions appear to be equally suitable. The stock culture was held in a refrigerator in nutrient agar (Difco) butts layered with sterile mineral oil. To prepare suspensions for the assay of nitrate, a three-step procedure was followed using conventional bacteriological techniques.

1. Six milliliters of nutrient broth (Difco or B.B.L.) placed in a screw-cap test tube are inoculated from a 24-hour agar slant culture. The tubes are incubated for 2 days at 30° C., or are placed on a reciprocating shaker at a 30° angle and shaken at room temperature for 8 to 12 hours. The tubes may be stored in a refrigerator for several months.
2. Approximately 100 ml. of nutrient broth in a 500-ml. bottle are inoculated with the growth from a single tube from step 1. The bottles are placed on a shaker at room temperature and shaken until good growth appears (usually 6 to 8 hours) and are then used immediately for step 3 or refrigerated for periods up to 2 months.
3. Depending upon the amount of bacterial suspension needed, large volumes of nutrient broth (500 to 1000 ml.) are inoculated in a 4-liter Erlenmeyer flask with the contents of one bottle from step 2, incubated overnight at 30° C., placed on a reciprocating shaker at room temperature, and shaken until the bacterial concentration reaches 0.6 to 0.8 mg. of cells (dry weight) per ml. The concentration of cells may be conveniently estimated by determination of the turbidity. Such measurements on a sterile system are facilitated by fusing an 18 × 150 mm. culture tube to the side of the flask about 3 cm. from the bottom and parallel to it. Turbidity measurements are made at 640 mμ using a similar 18-mm. tube containing nutrient broth to adjust the spectrophotometer or colorimeter to zero absorbance. The proper concentration of bacteria is indicated by absorbance values between 0.25 and 0.40.

Culture Media. Preparations of nutrient agar and nutrient broth are made according to the directions found in the Difco (*2*) or B.B.L. (*1*) manuals. The nutrient agar is prepared by suspending 23 grams of the dehydrated medium in 1000 ml. of cold, distilled water. The suspension is then heated to boiling until dissolution is complete. After distribution, the tubes are sterilized for 15 minutes at 15-pound pressure. The nutrient broth is prepared by dissolving 8 grams of the dehydrated medium in 1000 ml. of distilled water. After distribution, the bottles and tubes are sterilized for 15 minutes at 15-pound pressure. The nutrient broth used for adjustment of sample volume need not be sterilized.

Nitrite present in the system is determined according to the method of Saltzman as modified by Garner and coworkers (*4*).

Reagents. Stock reagent solution is prepared by dissolving 0.1 gram of *N*(1-naphthyl)-ethylenediamine dihydrochloride in 100 ml. of water.

Working reagent solution is prepared as follows: Five grams of sulfanilic acid are dissolved in 700 ml. of warm water. After cooling, 170 ml. of glacial acetic acid are added, followed by 20 ml. of the stock reagent solution. The final volume is adjusted to 1 liter.

Standard Nitrate Solution. The standard nitrate stock solution contains 1.000 gram of dried, reagent grade potassium nitrate per liter. A working nitrate solution is prepared by dilution of the stock solution 1 to 5 with distilled water so that each milliliter of the working solution contains 200 γ of potassium nitrate.

Preparation of Standard Curves. Standard curves, which must be prepared for each series of determinations, are obtained by adding, in duplicate, to 16 × 120 mm. plastic centrifuge tubes the following volumes of working nitrate solution: 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 ml. Sufficient distilled water is added to each tube to bring the total volume to 1 ml. Then 9 ml. of bacterial suspension are pipetted forcefully into each tube in order to mix the nitrate solution thoroughly with the cells. The tubes are then incubated at 30° C. for approximately 1.5 hours. The cells are sedimented by centrifugation. A 1-ml. aliquot of the supernatant liquid from each tube is transferred to an 18 × 150 mm. test tube. Two milliliters of freshly prepared nutrient broth are then added to each tube. Finally 5 ml. of the *N*(1-naphthyl)-ethylenediamine dihydrochloride working solution (*4*) are added forcefully from a pipet, or an all-glass syringe, to effect thorough mixing. After 20 minutes the absorbance of the solution in each tube is determined at 550 mμ, using the solution containing no potassium nitrate as a blank for setting the spectrophotometer to zero absorbance. No appreciable change in absorbance is observed even after several hours. Minor changes in dilution of the final solution may be necessary, depending on the particular spectrophotometer and size of cuvette used. The absorbance of the solutions prepared, according to the above directions, are in the proper range for use with a Beckman Model DU or a Coleman Jr. instrument having cuvettes with 10- and 15-mm. light paths, respectively, both of which were used in this study.

Analysis of Samples. Sample solutions containing varying amounts of nitrate are analyzed in a manner similar to that used for the preparation of the standard curve, except that 1 ml. of unknown solution or of tissue extract is mixed with 9 ml. of bacterial suspension. After centrifugation, 0.1-, 1.0-, and 3.0-ml. aliquots of supernatant solution are adjusted to 3.0 ml. by addition of nutrient broth before the 5-ml. portion of working color reagent is added to each tube. The concentration

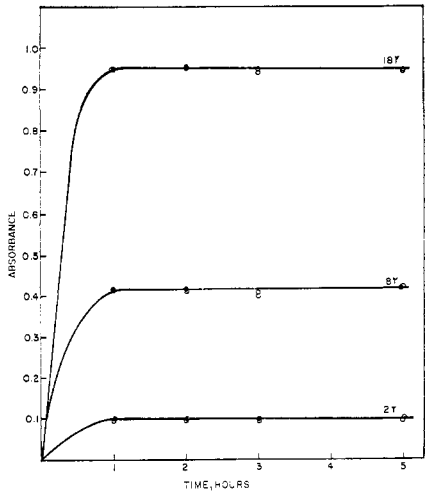

Figure 1. Influence of incubation time on reduction of nitrate to nitrite by *P. oleovorans* at 2, 8, and 18 γ of potassium nitrate per ml. of bacterial suspension

of nitrite in each tube is obtained from the standard curve. The amount of nitrite present in a sample before reduction of any nitrate may be determined and a correction made for it. In this case 9 ml. of nutrient broth are substituted for the bacterial suspension. All other steps remain the same as for the nitrate.

Preparation of Forage Samples. All forages are dried sufficiently to facilitate grinding. A 5-gram sample is suspended in a 500-ml. flask containing approximately 250 ml. of distilled water or 0.01*M* phosphate buffer at pH 7.0. After 30 minutes in the steamer, the suspension is diluted to 500 ml. and mixed thoroughly. It is not necessary to filter the extract before using.

Results

The reduction of nitrate to nitrite is quantitative and rapid. The nitrite, once formed, does not disappear upon prolonged contact of the sample with the bacterial suspension, even when incubated for as long as 20 hours. To determine the minimum time required for complete reduction of the nitrate, potassium nitrate was added at several levels to the bacterial suspensions. After incubation for various periods of time, one tube at each level was removed and assayed for nitrite content. Selected representative data are presented in Figure 1. At all levels the nitrate reduction occurs at a very rapid rate, and there is no decrease in the amount of measured nitrite over a 5-hour period. Further studies on the completeness of the nitrate reduction were made. Potassium nitrate was added at various levels to one set of tubes containing bacterial suspension. Sodium nitrite in equimolar amounts with respect to the potassium nitrate was added to a second set of tubes. Both sets of tubes were incubated for periods up to 20 hours at 30° C. and the nitrite present in each tube was determined according to the method just described. In addition, identical amounts of sodium nitrite were added to sterile nutrient broth and each tube was immediately analyzed for nitrite. The absorbances obtained for the tubes containing equimolar concentrations of nitrate or nitrite agreed well within the limits of experimental error in all three series of tubes, those with nitrate, nitrite, and nitrite in sterile broth.

Very good precision was obtained with this method of analysis. The data in Table I show the reproducibility which can be obtained, when the usual precautions employed in quantitative chemistry are observed.

The method described yields results comparable to those of the chemical method in most cases. Table II shows the results of analyses of various feedstuffs by both methods.

Table I. Precision of Analysis for Nitrate

KNO_3 γ/Ml. Bacterial Suspension	No. of Replicate Tubes	Supernatant Analyzed, Ml.	Range of Absorbance	Av. Recovery, γ KNO_3	Std. Dev., γ KNO_3
2	7	1.0	0.120–0.132	2.03	0.185
8	6	1.0	0.487–0.495	8.10	0.050
16	5	1.0	0.925–0.940	16.0	0.014
50	5	0.2	0.610–0.622	50.2	0.830
100	7	0.1	0.608–0.635	101.5	1.642

The sheep rumen microorganism is subject to inhibition by constituents of rumen fluid and of urine and correction must be made for this undesirable effect. Such inhibition by rumen fluid or urine is not observed in the case of *Pseudomonas oleovorans*, as shown in Table III.

The effect of the pH of the sample on the activity of the nitrate reductase was investigated. No difference in nitrate concentration could be detected in several different forages and urines when placed in buffer or in distilled water at pH values ranging from pH 4.2 to 7. This is due to the high buffering capacity of the bacterial suspension which maintained the pH value of the inoculum between 7 and 8. This method also gave excellent recoveries for nitrate from blood plasma to which additional potassium nitrate was added.

Discussion

The determination of nitrate by the method described allows quantitative determinations of the salt in samples containing between 0.1 and 10.0% nitrate as potassium nitrate, which is the range encountered in studies of nitrate toxicity. By further variation of dilutions, the range in nitrate content of samples which may be analyzed is greatly increased. The maximum amount of nitrate which has been found to be reduced in 1.5 hours by 9 ml. of bacterial suspension is more than twice the amount suggested in this paper.

The pH value of the extract appeared to be of little importance for the forages examined. Ensilage, a water extract of which results in a solution having a pH value in the neighborhood of 4, gave identical analyses for nitrate when extracted with both water and phosphate buffer at pH 7. However, if it is desired to determine nitrite present in the original samples, it would be desirable to neutralize the material before heating.

Table II. Nitrate Content of Various Animal Feeds

Sample	Potassium Nitrate, % This method[a]	Potassium Nitrate, % Chemical method[b]
Corn silage	0.42	0.34
Clover	0.29	0.32
	0.49	0.47
Corn silage	0.28	0.25
Native grass	0.47	0.46
Sorgo silage	0.36	0.35
Sudan grass	0.12	0.09
	0.12	0.25
Millet hay	4.9	4.3
Cane sorghum	7.5	7.8

[a] Average of 4 separate analyses.
[b] Average of 2 separate analyses.

When a variety of different samples in which the presence or absence of inhibitors is not known, are encountered, it is advantageous to take several aliquots of the original extract—viz., 0.1, 0.5, and 1.0 ml.—to which the bacterial suspension is added instead of the 1-ml. aliquot suggested. In this case, 3 ml. would be removed from each tube for color development. A linear relationship between nitrate found and the size of the aliquot would indicate the absence of an inhibitor.

Removal of bacterial cells by centrifugation, though a time-consuming step,

Table III. Effect of Rumen Fluid and Urine on Nitrate Reduction by Enzyme from *Pseudomonas oleovorans*

Mixture Assayed: Rumen fluid, ml.	Mixture Assayed: Water, ml.	KNO_3, γ per Ml.: Initially present	KNO_3, γ per Ml.: Added	KNO_3, γ per Ml.: Total recovered
9.0	1.0	...	...	6
9.0	1.0	6	100	109
4.5	5.5	3	100	108
1.0	9.0	0.6	100	105
Urine				
9.0	1.0	...	...	120
9.0	1.0	120	100	221
4.5	5.5	60	100	168
1.0	9.0	12	100	117

permits a blank having no turbidity and therefore a very low absorbance. Furthermore, if unsatisfactory results are obtained due to accidental spillage or to an excessively high or low nitrate content, it is not necessary to repeat the entire incubation process, but merely the color development step using a suitable aliquot of supernatant solution. If many similar samples in the same range of nitrate content are to be studied, it appears that the procedure of Garner and coworkers could be employed advantageously. It employs a smaller amount of bacterial suspension to which the color reagent is directly added.

The time involved in determination of nitrate by the method described varies with the number of samples to be analyzed. A standard curve must be prepared for each batch of samples, although it may not be necessary to determine all the points indicated, as the Beer-Lambert law is followed between 2 and 20 γ of nitrate. With a large number of samples the actual time required for media preparation, centrifugation, etc., amounts to less than 20 minutes per sample.

Acknowledgment

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PLANT GROWTH REGULATORS

Synthesis and Biological Activity of Some Quaternary Ammonium and Related Compounds That Suppress Plant Growth

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Certain quaternary ammonium and related compounds cause plants to grow short and sturdy, with intense green foliar coloration, and experimentally, they have prolonged the life of test plants, such as bean. The synthesis and chemistry of one of these compounds, Amo-1618, is described, as are the syntheses of structurally related compounds. Eight compounds were rated biologically as highly active; four were rated as moderately active, and nine were inactive inducing no biological response in young bean seedlings.

THE RELATIVELY HIGH DEGREE of potency of certain quaternary ammonium and other compounds (supplied by National Research Council, Chemical-Biological Coordination Center; synthesized by R. L. Shriner, State University of Iowa), which influence rate of growth, over-all size, and longevity of many kinds of plants, was discovered by Wirwillie and Mitchell (*14*), and the study was extended by Marth, Preston, and Mitchell (*9*). In a recent study using Amo-1618 on chrysanthemums, Cathey (*5*) found that, in addition to producing a retarding effect on growth, this compound was antagonistic to the growth accelerating effect of gibberellin.

The synthesis of one of the most important of the original compounds tested, Amo-1618 (2-isopropyl-4-dimethylamino-5-methylphenyl 1-piperidinecarboxylate methyl chloride), and of additional new compounds, some of which also induce plants to develop short, sturdy stems with intense green foliar coloration, is presented. The corresponding iodide, designated Amo-1619, has previously been reported (*14*) as highly active. A compound, VIII, isomeric with Amo-1619, prepared from less expensive carvacrol, is also active. (See Table I for compound names and Figure 1 for structural formulas.)

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Experimental

Preparation of Amo-1618 (I) from Thymol. 4-Nitrosothymol. Prepared from thymol (reagent grade, 3.33*M*) according to the method of Kremers, Wakeman, and Hixon (*6*). Yield 83.1%, m.p. 159–161° C.; reported 87%, 160–164° C.

4-Aminothymol. Prepared from 4-nitrosothymol (*6*) using 3.50*M* quantities. Yield 93.0%; reported 82.1%.

4-Dimethylaminothymol Methiodide. 4-Aminothymol (528 grams, 3.19*M*) was added to anhydrous methanol