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Methods for Bacteriological Examination of Soils
Media for Quantitative Determination of Bacteria in Soils

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METHODS FOR THE BACTERIOLOGICAL EXAMINATION OF SOILS

BY PERCY EDGAR BROWN.

Because the idea of examining soils bacteriologically owed its inception to the development of the gelatine plate method for isolating pure cultures, very naturally the first investigations of soil bacteria were by the use of plate cultures. The studies, therefore, were purely quantitative in nature. The results secured by the pioneers in this work demonstrated some facts of considerable interest, but it soon became evident that mere quantitative determinations of the bacteria in soils were inadequate to give information regarding the relation of such organisms and their activities to the fertility of the soil.

The media available permitted of the development of only a few of the organisms normally present in soils, and the results secured gave no indication of the chemical changes occurring in the soil through the agency of bacteria. Moreover, the fact was clearly recognized that the importance of bacteria in determining the fertility of a soil depended on their ability to transform the complex animal and plant residues in the soil into forms available for plant nourishment. Consequently, the early investigators turned their attention very largely toward the study of methods of determining the physiological activities of the groups of soil organisms which accomplished the solution of complex compounds. From the fact that nitrogen seemed most likely to be lacking in soils, the groups of bacteria which change insoluble proteins through several stages into soluble, available nitrates were the first to be examined. Thus the ammonifying and nitrifying bacteria have received considerable attention. The nitrogen fixing organisms, playing so important a role in placing the inexhaustible supply of nitrogen in the atmosphere at the disposal of plants, and the denitrifying bacteria, at one time supposed to be of particular significance in causing losses of nitrogen from the soil, have also been studied.

Remy made the first attempt to study systematically methods for measuring the activities of special groups of soil organisms. He devised nutrient solutions of such composition that certain groups of organisms were favored to the exclusion of all others, and the products of the activities of these groups were then measured chemically.

Many experiments have since been made, using Remy's solu-

[1]Voorhees & Lipman, Bull. 194, Office of Expt. Stats. U. S. Dept. Agr., 1907.

tion method, and these are discussed so exhaustively in another publication[1] that they need not be considered here.

DIFFICULTIES IN USE OF SOLUTIONS

The difficulties attendant upon the use of solutions are considerable. In the first place the physical conditions in solutions are so entirely different from those in soil that the bacteria introduced, whether in soil or in soil infusions, may be greatly influenced. The growth of many species is undoubtedly prevented; the transference of the bacteria into the solutions may cause plasmolysis; reduction of virulence or physiological efficiency of important groups may occur, and species relationships will be entirely altered. Furthermore, the chemical composition of the soil is left out of account entirely and this has been shown to be of considerable importance in the study of bacterial activities in a soil.[2]

There is also the difficulty that small quantities of soil used for inoculation may not have the same properties as one hundred grams of fresh soil.

The studies of Stevens and Withers[3] showed quite conclusively that the activities of various groups of organisms are quite different in soils from what they are in solutions, and they concluded that no "adequate knowledge of the efficiency of soil organisms in effecting chemical changes can be attained by tests in solutions."

Although some investigators, notably Löhnis,[4] still cling to the solution method and advocate its use, most of the experiments now conducted are carried out by using soil itself as a medium according to the suggestions of Stevens[5] and Withers and Lipman and Brown.[6] The contention of Löhnis, that because decomposition processes going on in the soil take place in the soil water, therefore tests in solution are an adequate measure of such destructive processes, seems hardly warranted by the evidence at hand.

Sand has been suggested as a medium, but experiments have shown[7] it to be quite unsatisfactory, particularly in ammonification studies, as ammonia is lost very rapidly by volatilization.

SOIL ITSELF THE MOST RATIONAL MEDIUM

The soil itself, therefore, seems to be the most rational medium to be employed to measure the extent of bacterial activities occurring therein.

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[2]Lipman & Brown, Centbl. f. Bakt. 2 abt Bd. 26 (1910) p. 590.
[3]Bull. 132, Bur. Chem. U. S. Dept. Agr., p. 34.
Centbl. f. Bakt. 2 abt. 23 (1909) p. 776.
Centbl. f. Bakt. 2 abt. 27 (1910) p. 169.
[4]Landw. Jahr. Bd. 42 Heft 5 (1912) p. 751.
[5]Science in Ser. 27 (1908) No. 704, p. 991.
[6]New Jersey Stat. Rpt.—1908—p. 129.
[7]Lipman, Brown & Owen, Centbl. f. Bakt. 2 abt 30 (1911) p. 156.
Lemmerman, Fischer, etc., Landw. Jahr. 38 (1909), p. 319.

Many important questions immediately arise in considering the use of soil as a medium, and chief among these is the condition in which the soil should be used.

Sterilized soil, air-dry soil, or fresh soil may be employed, the two former being inoculated with fresh soil infusions, the latter allowing the organisms normally present to perform their natural functions.

Soil may be sterilized in the autoclave by steam under pressure, or by the use of various antiseptics. In the first case, great difficulty is experienced in obtaining complete sterilization of a large quantity of soil; furthermore, there may occur a transformation into more easily decomposable form and possible loss of nitrogen compounds, and the rearrangement of mineral compounds, changing entirely the chemical character of the soil. It has been shown[8] that sterilized soils subsequently inoculated do not recover completely their characteristic power of decomposing nitrogenous organic matter. In the case of humus soils, sterilization increases the amount of nitrogen driven off in distillation with magnesia. The same objections pertain to the sterilization of the soil by means of antiseptics, particularly if, as is usually the case, the soil is rinsed with water to insure complete removal of the antiseptic. Furthermore, the physical properties of the soil, particularly the water-holding power, are vitally affected and acid products may be formed in considerable amounts.

When air-dry soil is employed, the greatest objection, of course, is that it is only partially sterile and the species relationships are totally different from those in the corresponding fresh soil. There is also the objection that air-drying affects the chemical and physical character of the soil. The authors just cited[9] have shown furthermore that drying changes the power of soil bacteria to accomplish their normal functions; for example, they found the nitrifying power considerably reduced but the denitrifying power unaffected.

Infusions of soil which must be used to inoculate both the sterilized and dried soils are objectionable for the reasons already mentioned.

THE ADVANTAGES OF FRESH SOIL.

Fresh soil, possessing none of the disadvantages mentioned above, offers, therefore, conditions as closely approximating field conditions as possible: the chemical, physical, and bacteriological conditions remain unaltered from field conditions at the beginning of the experiment. There is, of course, the difficulty of obtaining representative samples, but that may be accomplished

[8] Lemmermann, Fischer, etc., Landw. Jahr. 38, 1909, p. 319.
[9] Lemmermann, Fischer, etc., l. c.

by great care in taking the samples, thorough mixing, and frequency of sampling. The method which the author has employed with considerable success is completely described in a previous publication.[10]

There is the additional objection that the securing and mixing of the sample aerates the soil so thoroughly that increased bacterial activities may occur. This is probably the case, and if it were not for the necessity of thorough mixing in order to obtain representative samples, portions of soil could be cut out and transported to the laboratory for experimentation with little or no aeration occurring. As the matter stands, however, it is an open question which method is the more advisable, no comparisons having as yet been made.

As has already been noted, most of the investigators studying the physiological activities of bacteria in soils are agreed that fresh soil is the medium which should be employed.

The next question which arises is the choice of materials which must be added to soils when tested in the laboratory in order to permit of the accumulation of products of bacterial activities in sufficient quantities to be chemically estimated. For nitrification, ammonium sulfate has been commonly employed and has proved quite satisfactory. It may be applied in solution and hence is suitable for use with fresh soil. For azofication, mannite and dextrose, both of which may be added in solution have proven to be the best of the sugars in permitting the fixation of nitrogen.

The most difficulty has been experienced in obtaining some substance which would be perfectly satisfactory for measuring the ammonifying power of a soil. Peptone proved quite unsatisfactory, which was attributed to enzyme action. Other nitrogenous materials such as urea, albumen, cottonseed meal and dried blood, have been employed in the work of the various investigators already mentioned. Urea was found to be subject to the same objection as peptone; albumen caused difficulty because of its coagulation on sterilization; the other materials, cottonseed meal and dried blood, gave indications of greater value. The objections to both of these substances are the varying physical and chemical composition of different samples. It has been found that comparisons are difficult, in the case of dried blood for example, without employing, not only the same period of incubation and the same temperature, but also dried blood of the same composition. There is always to be considered the change sterilization causes in the composition of different samples; the difficulty of mixing thoroughly such materials with fresh soils, with the consequent impossibility of obtaining satisfactory dupli-

[10]Research Bull. No. 5—Iowa Expt. Stat., Feb., 1912.

cate determinations, and finally, the difficulty in distillation due to foaming.

Considering all the facts just mentioned, the writer has attempted to eliminate some of the objectionable features of the methods from which at present investigators must choose. Realizing that the use of fresh soil presents conditions with the least objections, attention has been centered on its use and comparisons made of the results secured with those obtained through the use of dry soil inoculated with infusions. The use of a new material, casein, has also been tested and its use as a measure of ammonification has given indications of considerable value.

SERIES A.—Use of Egg Albumen to Measure Ammonification in Fresh and Air-Dry Soil.

This series was planned to test the use of egg albumen as a measure of ammonification in fresh and air-dry soil. Four 100 gram quantities of fresh soil were weighed out and 10 c.c. of a sterile 20 per cent albumen solution (= 2 gms. albumen) were added to each.

The moisture content of the soil was found to be 20.00 per cent. Thus the final moisture content with the material added was 30.00 per cent, just about the optimum for the soil employed. Four 100 gram quantities of air-dry soil from the same source were weighed out and 10 c.c. of a 20 per cent solution of albumen added. Then 20 c.c. of a fresh infusion of the same soil were added to each. This infusion was prepared as usual by adding 100 grams to 200 c.c. of sterile water and shaking for five minutes. The moisture content of these samples was therefore the same as in the case of the fresh soil. Both lots were incubated for five days at room temperature.

The results are given in table I:

TABLE I.

Lab. No.	Fresh Soil		Lab. No.	Air-dry Soil	
	Ammonia mgs N.	Av. mgs N.		Ammonia mgs N.	Av. mgs N.
1	90.64		5	40.98	
2	89.85		6	42.56	
3	95.76		7	39.01	
4	91.03	91.82	8	41.38	40.98

Glancing over these results we find that the determinations agreed very satisfactorily and the averages of the four results show that the ammonification of the albumen proceeded much

more rapidly in the fresh soil than in the same soil air-dried and inoculated with a bacterial suspension from a fresh sample. The reason for this may be attributed to the change in the physical or chemical composition of the soil on air-drying or more likely to the fact that the species relationships in the air-dry soil, which were undoubtedly considerably altered in the process of air-drying, caused development of the bacteria introduced in the soil infusions along different lines from that which would normally occur in fresh soils.

The egg albumen solution is shown to be fairly satisfactory and a decided improvement over the dried blood as it is practically impossible to mix the latter material with fresh soil thoroughly enough to secure satisfactory duplicates. The chief objections to the albumen is the difficulty of preparing a solution of it. This must be brought about in the cold, using sterile water, and considerable time is required to accomplish complete solution.

The much more rapid ammonification evidenced in this series when fresh soil was used beyond that brought about in air-dry soil made it desirable to determine whether the same differences in ammonifying power between field soils would be shown by the use of fresh and air-dry soils.

SERIES B.—Egg Albumen to Measure Ammonification in Fresh, Air-Dry and Sterilized Soil.

This series was planned to test the use of egg albumen as a measure of ammonification in fresh, air-dry and sterilized soil, and also to compare the results obtained with this material with those secured by using dried blood. The soils from three plots differently treated were used to determine whether the same differences would be apparent by the different methods.

The plots from which the samples were drawn were numbered and treated as follows:

Plot 510—Check
Plot 509—2 T ground limestone applied in 1911
Plot 508—3 T ground limestone applied in 1911.

The plots were all in corn during the season of the experiment.

Fresh soils were drawn from these plots with the usual precautions and 100 gram quantities weighed off and 10 c.c. of a 20 per cent albumen solution were added to each, the moisture content was determined and this was then adjusted in the samples to 30.00 per cent.

Air-dry soil from these plots was secured and albumen solution added to 100 gram portions, as before, and 20 c.c. of infusions of fresh soils were added. Air-dry soil sterilized in the

autoclave was also employed, albumen solution and infusions being added as previously. These samples were then all incubated for six days at room temperature.

Tumblers were used for the fresh and air-dry samples, but the sterilized samples were kept in 500 c.c. Erlenmeyer flasks.

One hundred gram quantities of fresh soil similarly obtained received additions of 5 grams of dried blood, which was thoroughly mixed in, the moisture content was adjusted to 30.00 per cent, 75 per cent being figured the optimum for the organic matter, and the incubation period was seven days.

Air-dry soils from the plots were weighed off in 100 gram quantities, five grams of dried blood thoroughly mixed in, and 20 c.c. of infusions of the fresh soils were added. Again the incubation period was seven days at room temperature.

The moisture in the fresh soils was as follows:

510—20.00%
509—17.50%
508—15.00%

The results obtained in this series are given in table II:

TABLE II.

THE AMMONIFICATION OF ALBUMEN—SIX DAYS.

Plot No.	Fresh Soil			Air-dry Soil+ Infusions from fresh soils			Sterilized Soil+ Infusions from fresh soils		
	Lab. No.	Ammonia mgs N.	Av. mgs N.	Lab. No.	Ammonia mgs N.	Av. mgs N.	Lab. No.	Ammonia mgs N.	Av. mgs N.
510	1	65.81		9	59.90		15	59.50	
	2	71.72	68.76	10	59.90	59.90	16	64.24	61.87
509	3	83.70		11	70.93		17	66.20	
	4	88.27	87.48	12	65.81	68.37	18	72.12	69.16
508	5	103.64		13	76.06		19	74.87	
	6	104.43	104.03	14	70.14	73.10	20	75.27	75.07

THE AMMONIFICATION OF DRIED BLOOD—SEVEN DAYS.

Plot No.	Air-dry Soil+ Infusions of fresh soils			Fresh Soil.		
	Lab. No.	Ammonia mgs. N.	Av. mgs. N.	Lab. No.	Ammonia mgs. N.	Av. mgs. N.
510	21	89.06		27	125.71	
	22	89.85	89.45	28	129.65	127.68
509	23	96.94		29	137.55	
	24	99.31	98.12	30	133.99	135.77
508	25	104.83		31	145.02	
	26	103.25	104.04	32	142.66	143.84

Several interesting points are evident in the table:

In the first place when albumen was used, just as in series A, larger amounts of ammonia were produced when the fresh soil

was employed than with air-dry or sterilized soil.

Furthermore, the greatest differences in the ammonifying power of the soils from the different plots were given in the fresh soil samples although the effects of the treatment were shown both with the air-dry and sterilized soils. The amounts of ammonia produced when the air-dry and sterilized soils were employed were practically indentical, and the same differences in the soils were evidenced. The amounts of ammonia produced with the sterilized soils were probably larger than they would have been in beakers for they were in Erlenmeyer flasks and consequently had a larger surface exposed to the air.

The ammonification of the dried blood in fresh soil was greater than in the air-dry samples, but the differences in the ammonifying power of the soils from the different plots were very slightly more pronounced.

Comparing the results secured using dried blood and albumen we find that the latter material permitted of a more distinct differentiation of the soils of the plots according to their ammonifying power. The amounts of ammonia were also smaller, due largely to the slightly shorter period of incubation and the difficulty in distilling was considerably lessened.

It is interesting to note that these plots were those which were used the previous season as a basis for the study of the effects of liming on field soils and that the effects noted in that work[11] were confirmed here, the ammonifying power of the soil being much enhanced by the application of limestone. Furthermore, the differences were even more pronounced than they were the previous season, immediately following the application.

The agreement of duplicate determinations in this series is fair, in general the results obtained when using the albumen being much better than those secured when using the dried blood.

The difficulty, already mentioned, which is encountered when albumen is used is very annoying and the time required to bring about a solution of the albumen is considerable, hence it seemed to the writer that a material which could be more easily dissolved would be much preferable, and casein suggested itself.

The following series was planned to determine the suitability of casein as a measure of ammonification.

SERIES C.—Use of Casein Solution as a Measure of Ammonification.

In this series a solution of casein was prepared, dissolving 20 gms. in 200 c.c. of water and sodium hydrate was added

[11]Brown, Research Bull. 5, Iowa Expt. Stat., 1912.

until solution was complete and the solution was then sterilized in the autoclave. Ten c.c. of this solution was added to each of six 100 gram portions of fresh soil drawn from the three plots already described and weighed off in duplicate in tumblers. The moisture content of the soils was determined and found to be as follows:

Plot 510—20.00%
Plot 509—24.00%
Plot 508—20.00%

Enough sterile water was then added to the soils from plots 510 and 508 to make them correspond with that from plot 509, the content of the samples then being 34.00 per cent or somewhat above the optimum.

The samples were incubated for three days at room temperature and then analyzed with the following results:

TABLE III.

Plot No.	Lab. No.	Ammonia mgs N.	Av. mgs N.
510	1	23.64	
	2	23.25	23.44
509	3	32.71	
	4	33.49	33.10
508	5	39.41	
	6	40.19	41.80

These results show that casein may be used very satisfactorily in this work and the differences which with other materials appeared in the soils of these plots were apparent also with casein.

Further tests of this substance seemed very desirable and the following series were planned with this in mind.

SERIES D.—Use of Casein with Fresh, Air-Dry Standard and Air-Dry Special Soils.

Fresh soils were obtained from the plots and the moisture content determined. This was found to be:

Plot 510—20.00%
Plot 509—20.00%
Plot 508—19.50%

One hundred gram portions were weighed off as usual and 10 c.c. of casein solution added. This solution was prepared more carefully than was the case in the preceding series as there was thought to be danger of using a slight excess of sodium hydrate and thus affecting the bacteria in the soil when the solution was added. One hundred grams of casein were dissolved in 1,000 c.c. of water containing 70 c.c. of normal sodium hydrate, solution being complete and sterilization performed in

the autoclave. The moisture content of the samples was adjusted to 30.00 per cent.

It was thought advisable also to include in this series a comparison of the use of a standard air-dry soil inoculated with infusions and air-dry soils from the special plots inoculated with infusions with fresh soil and with each other. Consequently a sample of soil from an untreated plot was obtained and air dried, and 100 gram portions of it weighed off in tumblers; 10 c.c. of the casein solution prepared as described were added and 20 c.c. of 5-minute infusions of fresh soils from the plots were added. A moisture content of 30.00 per cent was thus secured. Similarly two one hundred gram portions of air-dry samples of soil from each of the three plots were weighed off, 10 c.c. of casein solution added and 20 c.c. of 5-minute infusions of fresh soils from the same plots were added. All these samples were incubated for three days at room temperature.

A comparison series was started at the same time using fresh soil, air-dry standard soil and air-dry special soils, with dried blood added. The moisture content of the fresh soil samples was adjusted to 30.00 per cent, 75 per cent being allowed as the optimum for the dried blood. Infusions of fresh soils were used with the air-dry standard and air-dry special soils and the moisture content of these brought up to 30.00 per cent.

The incubation period for these samples was six days at room temperature, which has been found to be the optimum period.

The results obtained upon distillation of the ammonia formed in the samples with both the casein and dried blood are given in table IV.

In looking over the results given in the table several facts stand out very distinctly. In the first place it is evident that the casein solution was quite satisfactory as a measure of ammonification. It is also clear that the casein solution permitted of a greater differentiation in the soils of plots differently treated than the dried blood. The amounts of ammonia produced were smaller and there was less difficulty therefore in the distillation. The duplicate determinations agreed remarkably well, much better than it is possible to make the determinations with dried blood agree.

Comparing the results secured using fresh soil, air-dry standard soils and air-dry special soils, the differences in the ammonifying power of the three soils were shown much more distinctly with the fresh soil when either casein or dried blood was used.

The air-dry special soils showed somewhat greater differences from the air-dry standard soils. Evidently the chemical composition of the soil was of considerable importance as has already been mentioned.

Another fact which is quite noticeable is the excellent agree-

TABLE IV.

THE AMMONIFICATION OF CASEIN—THREE DAYS.

Plot No.	Fresh Soil			Air-dry Standard Soil+ Infusions of fresh soils			Air-dry Special Soil+ Infusions of fresh soils		
	Lab. No.	Ammonia mgs N.	Av. mgs N.	Lab. No.	Ammonia mgs N.	Av. mgs N.	Lab. No.	Ammonia mgs N.	Av. mgs N.
510	1	24.32		7	33.35		13	27.08	
	2	23.15	23.73	8	32.96	33.15	14	25.90	26.49
509	3	30.61		9	38.06		15	31.00	
	4	31.39	31.00	10	38.85	38.45	16	30.61	30.80
508	5	45.71		11	41.99		17	43.35	
	6	44.92	45.31	12	41.20	41.59	18	42.57	42.96

THE AMMONIFICATION OF DRIED BLOOD—SIX DAYS.

Plot No.	Fresh Soil			Air-dry Standard Soil+ Infusions of fresh soils			Air-dry Special Soil+ Infusions of fresh soils		
	Lab. No.	Ammonia mgs N.	Av. mgs N.	Lab. No.	Ammonia mgs N.	Av. mgs N.	Lab. No.	Ammonia mgs N.	Av. mgs N.
510	19	107.91		21	153.82		27	144.80	
	20	112.23	110.07	22	151.47	152.64	28	140.49	142.64
509	21	129.10		23	160.49		29	152.25	
	22	125.18	127.14	24	163.63	162.06	30	158.53	155.39
508	23	153.18		25	170.30		31	172.26	
	24	155.39	155.78	26	167.55	168.92	32	167.95	170.10

ment of the duplicate determinations when the casein was used. In fact, the duplicates agree like a chemical determination and this is in striking contrast with the large variations which frequently occur in the duplicates when dried blood is employed.

Furthermore, the distillation is a much simpler process. The foaming which is so troublesome with the dried blood is very much reduced and causes little trouble. The short incubation period of the casein treated soils is also a point of considerable importance, as in formulating a practical method the time element is an important factor in determining its value.

SERIES E.—Use of Fresh Soils with Adjusted and Unaltered Moisture Content.

When fresh soils are employed, the problem of the proper regulation of the moisture conditions for optimum bacterial activities must be faced.

The variation in moisture content of different plots of the same series of experiments may be quite large and the question arises whether in testing the soils the moisture should be allowed to remain as it is in the field, or whether it should be adjusted to the optimum. In the preceding series, the latter method was followed and it is undoubtedly the most rational method as the

ammonifying power of a soil means the power of the soil to produce ammonia under optimum conditions.

Although the moisture content of soils may vary considerably from plot to plot in the same series, in field plots carefully laid out, as they should be, on uniform land, well drained, etc., the variations in moisture content may be slight. It was deemed advisable therefore to determine whether slight variations in moisture conditions would materially affect the results. Fresh soils were consequently drawn from the three plots used in the preceding series and the moisture content determined as previously by drying carefully in the hot air oven. One hundred gram quantities of the fresh soils were weighed off and 10 c.c. of casein solution added to each.

In duplicates of each soil the moisture content of the soil was adjusted to the optimum at 30.00 per cent, while in other duplicates of the same soil no adjustment was made.

The moisture content of the fresh soils was:

Plot 510—20.00%
Plot 509—19.00%
Plot 508—18.50%

The samples were incubated for three days at room temperature and then the ammonia formed was distilled and collected in the usual way.

The results obtained are given in table V:

TABLE V.

Plot No.	Moisture Content Adjusted to the Optimum.			Moisture Content Unaltered		
	Lab. No.	Ammonia mgs N.	Av. mgs N.	Lab. No.	Ammonia mgs N.	Av. mgs N.
510	41	23.75		47	22.96	
	42	22.96	23.35	48	22.18	22.57
509	43	34.92		49	34.92	
	44	34.14	34.54	50	35.32	35.12
508	45	38.85		51	39.63	
	46	39.63	39.24	52	38.85	39.24

The results given in this table show that a variation in moisture content of one and one half per cent (1½%) seemed to have no influence on the differences in the ammonifying power of plots differently treated. In fact, the figures obtained are almost identical.

It is evident therefore that normal differences in the moisture content of field plots well laid out on level ground seldom exceeding two per cent are negligible.

From the facts however, that in planning field plots it is extremely difficult often to have just the ideal location and that much larger differences in moisture than two per cent may occur,

it is believed that for ammonification tests the water content of the soil should be ascertained and sterile water added to bring it up to the optimum. For instance in this series of experiments plot 510 always contained slightly more water than the other two, a difference of 5 per cent. showing in Series A. Plot 510 is on lower ground and consequently would have a tendency to be more moist.

Unfortunately it was not possible to test the effect of larger variations than two per cent of moisture on the ammonification of casein, but it is a safe assumption that quite appreciable effects might be evidenced by differences of more than 5 per cent.

The results secured in this series with the casein check those of the preceding series and again we note the remarkable agreement of the duplicate determinations.

SERIES F.—Use of Casein in Smaller Amounts and With Shorter Incubation Period.

One further point was considered worthy of testing and that was whether it would be feasible to employ smaller amounts of casein and shorten the incubation period, and secure satisfactory results. As has been noted the time factor is of considerable importance in such experiments, and the shorter the necessary period of incubation, the better.

A further test was also included comparing the use of fresh soil with air-dry standard and air-dry special soils. Tests of a smaller amount of albumen solution and shorter period of incubation were also carried out in a similar way, and for the sake of comparison tests using dried blood were made as usual.

The fresh soils were drawn as previously to one lot of the three soils in duplicate, 5 c.c. of the casein solution were added, to another 5 c.c. of a 10 per cent albumen solution were added to the third lot 5 grams of dried blood.

The moisture content of the fresh soils was determined and found to be as follows:

Plot 510—18.50%
Plot 509—17.00%
Plot 508—16.50%

All the fresh samples received additions of sterile water to bring the content up to 30.00 per cent.

The infusions were prepared as usual and added to the air-dry samples. The samples receiving casein were incubated for two days, those receiving albumen three days, and those with dried blood six days, the optimum periods for each.

The results are given in table VI:

TABLE VI.

THE AMMONIFICATION OF CASEIN—TWO DAYS.

Plot No.	Fresh Soil. Lab. No.	Ammonia mgs N.	Av. mgs N.	Air-dry Standard Soil+ special infusions Lab. No.	Ammonia mgs N.	Av. mgs N.	Air-dry Special Soil+ special infusions Lab. No.	Ammonia mgs N.	Av. mgs N.
510	107	7.46		113	8.4		119	9.42	
	108	8.63	8.04	114	10.59	9.41	120	9.42	9.42
509	109	11.38		115	9.42		121	10.59	
	110	11.38	11.38	116	11.38	10.40	122	11.38	10.98
508	111	13.73		117	12.16		123	(lost)	
	112	15.30	14.51	118	13.34	12.75	124	13.73	13.73

THE AMMONIFICATION OF ALBUMEN—THREE DAYS.

Plot No.	Fresh Soil Lab. No.	Ammonia mgs N.	Av. mgs N.	Air-dry Standard Soil+ special infusions Lab. No.	Ammonia mgs N.	Av. mgs N.	Air-dry Special Soil+ special infusions Lab. No	Ammonia mgs N.	Av. mgs N.
510	125	3.92		131	13.73		137	9.03	
	126	4.42	4.17	132	12.95	13.34	138	(lost)	9.03
509	127	7.46		133	16.48		139	11.38	
	128	7.46	7.46	134	16.09	16.23	140	11.38	11.38
508	129	10.99		135	10.01		141	19.23	
	130	10.20	10.59	136	(lost)	20.01	142	17.27	18.25

THE AMMONIFICATION OF DRIED BLOOD—SIX DAYS.

Plot No.	Fresh Soil Lab. No.	Ammonia mgs N.	Av. mgs N.	Air-dry Standard Soil+ special infusions Lab. No.	Ammonia mgs N.	Av. mgs N.	Air-dry Special Soil+ special infusions Lab. No.	Ammonia mgs N.	Av. mgs N.
510	143	105.16		149	154.21		155	144.40	
	144	116.15+	110.65	150	151.07	152.64	156	145.97	145.18
509	145	12 .39		151	158.92		157	160.10	
	146	136.56	130.47	152	163.24	161.08	158	149.90	155.00
508	147	151.86		153	169.52		159	175.79	
	148	155.39	153.62	154	171.87	170.69	160	175.01	175.40

The results obtained here demonstrate again the fact that fresh soil allowed of the greatest differentiation in the soils of the plots.

Furthermore, the casein and albumen both gave better results than the dried blood; that is, they showed greater differences between the soils than did dried blood.

Comparing the results using air-dry standard soil inoculated with special infusions and air-dry special soils similarly inoculated, we find that the latter method showed greater differences in the soils although the former showed differences which were in the same direction.

The smaller amount of casein and albumen used here seem

hardly as satisfactory as the larger amount previously employed. Again the advantages of the casein are shown by the good agreement of duplicate determinations. Albumen, as has been noted, gives good results, but the difficulties attendant upon the preparation of the solution are a distinct detriment to its use.

CONCLUSIONS.

1. Fresh soil is the most rational medium for studying the physiological activities of soil bacteria.

2. Fresh soil permits of the greater differentiation in field soils, differently treated, according to the activities of the ammonifying bacteria than air-dry soils inoculated with infusions.

3. Air-dry soils from particular plots inoculated with infusions of fresh soils show greater differences than an air-dry standard soil inoculated with infusions from the various soils. Evidently the chemical character of the soil is of considerable moment when considering its ammonifying efficiency.

4. An albumen solution may serve as a measure of ammonifiaction and is preferable to dried blood, but it possesses the disadvantage that it is very difficult to prepare.

5. A solution of casein proved quite satisfactory as a measure of ammonification possessing none of the disadvantages of the other nitrogenous materials, and allowing of greater differentiation among different soils.

6. Slight variations in moisture conditions in field soils do not seem to affect materially the ammonification of casein. It is advocated, however, that in view of the large variations which may occur in the moisture content of field soils, that the conditions be adjusted to the optimum in the tests.

7. Ten c.c. of a 10 per cent solution of casein is found to be the best amount to use per 100 gms. of fresh soil and the optimum incubation period at room temperature is three days.

MEDIA FOR THE QUANTITATIVE DETERMINATION OF BACTERIA IN SOILS

The great variety of organisms present in the soil, all of interest from the fertility standpoint, precludes absolutely all possibility of ever formulating a medium which will permit of the growth of all species. For instance, it is clearly impossible under the artificial conditions inherent in laboratory methods to encourage the growth of aerobic and anaerobic species together, or to favor the nitrogen transforming bacteria and not discourage the nitrogen fixing organisms.

Our aim, therefore, is first to obtain a medium which will permit of the development of the maximum number of soil organisms and eventually so to modify such a medium that the bacteria developing on it will be limited to certain particular species which it is desired to study.

The work which has been done in the past has been confined to attempts to solve the first phase of this problem and the experiments which are reported here are a further contribution to the subject.

The media first employed in the quantitative examination of soils were bouillon gelatin and bouillon agar, each of which possessed certain advantages, but also presented certain objectionable features. Gelatin permits of the differentiation of certain types of organisms, as, for example, the liquefying, nonliquefying, and Streptothrix species of Hiltner and Störmer, but it has the disadvantage that the plates are quickly spoiled by the rapid growth of the liquefiers, and furthermore its high nitrogen content is an additional objection to its use. It is true that the first of these objections has been largely removed by the employment of the silver nitrate pencil, but not all investigators have been successful in the use of this preventive mechanism. Bouillon agar permits of the development of a greater number of organisms than gelatin but counts are often rendered impossible by the appearance of so-called "spreaders" which grow rapidly and soon cover the entire plate.

Soil extract gelatin and agar have been repeatedly suggested in the past and are occasionally employed now, but the objection to them is that a culture medium should be of such a composition that the results obtained by its use in one locality should be comparable with those obtained anywhere else, and soil extracts are obviously as variable as soils themselves.

A synthetic agar was suggested by Lipman and Brown[1] which contained small amounts of mineral salts and dextrose with

[1]New Jersey Stat. Rpt. 1908, pg. 132.

potassium nitrate as the nitrogen source, and the numbers of bacteria developing on this medium were far in excess of those appearing on comparative bouillon agar plates. Modifications of this medium have been tested by the same investigators[2] with ammonium sulfate or peptone substituted for the potassium nitrate with varying amounts of these materials, and with varying reactions, and the results have shown that the medium containing 0.05 gram of peptone per liter and of a reaction 0.5 per cent acid gave the largest numbers of colonies with all the soils examined.

SERIES 1.

The exact composition of this "modified synthetic" agar which will be used as a basis of comparison for the results secured with other media, is as follows:

I. Modified Synthetic Agar.
1,000 c.c. distilled water.
0.5 gm. K_2HPO_4.
0.2 gm. $MgSO_4$
0.05 gm. Peptone.
10.0 gms. Dextrose.
Trace $Fe_2(SO_4)_3$.
15.0 gms. Agar.

It seemed possible that by further modifying this medium still greater numbers of organisms might be encouraged to develop and with this idea in mind, the following experiments were begun. As the form in which the nitrogen exists seems to be of considerable importance in determining the value of the medium, it was decided first of all to ascertain the effect of substituting various nitrogenous substances for the peptone of the modified synthetic agar. According to this idea the following media were prepared:

II. Urea Agar.
Same as I. except that 0.05 gm. of urea was substituted for the peptone.

III. Asparagin Agar.
Same as I. except that 0.05 gm. asparagin was used instead of the peptone.

IV. Casein Agar, (A).
Same as I. except that 0.10 gm. casein replaced the peptone.

V. Albumen Agar, (A).
Same as I. except that 0.10 gm. albumen was employed instead of the peptone.

A modification of the albumen agar was also prepared supplying a large quantity of that material and omitting the dextrose.

VI. Albumen Agar, (B).
Same as I. except that the dextrose was omitted and 10.0 gms. of albumen were used instead of the peptone.

In each of these media, the nitrogenous materials were added

[2]Centralblatt f. Bakt. Abt. II, Bd. 25, 1910, pg. 447.

in solution to the agar just previous to sterilization in order to avoid boiling. Sterilization was performed in flowing steam and in the case of the albumen and casein media coagulation of these materials occurred. This, however, did not prove a serious detriment to the media as the coagulum was easily broken up by shaking the tubes vigorously before pouring the contents on the inoculum in the Petri dishes and no difficulty was experienced from this source in the counting.

Two media proposed by Temple[3] were also included in the experiment for the purpose of comparsion. They were made up as follows:

VII. Temple's Peptone Agar.

1,000 c.c. tap water.
1.0 gm. K_2HPO_4.
1.0 gm. Peptone.
15.0 gms. Agar.

VIII. Temple's Soil Extract Agar.

100 gms. soil in 1,000 c.c. water.
Boil and filter.
1.0 gm. K_2HPO_4.
10.0 gms. Peptone.
15.0 gms. Agar.

These media were prepared and sterilized in the autoclave in the usual way.

A modification of this latter medium was made, supplying only a small amount of peptone on the theory that some of the nitrogenous material present in the soil extract would help to satisfy the requirements of the bacteria.

IX. Temple's Soil Extract Agar, Modified.

Same as VIII. except that 0.05 gm. peptone replaced the 10.0 gms.

In order to obtain some substance more closely resembling the nutrient-materials present in the soil, it was decided to attempt the preparation of artificial humus and employ this as the source not only of the nitrogen but also of the carbon in the medium. Artificial humus has been repeatedly prepared in the laboratory by treating sugar with sulfuric acid but the product is incorrectly considered humus for it contains no nitrogen. This method of preparation was therefore of no use for the present purpose. After considering the various substances which might be employed, oats straw which is rather uniform in composition was chosen. It was treated with sulfuric acid and the excess of acid then thoroughly removed by washing and the dried material extracted with sodium hyroxide.

The extract obtained in this way was analyzed and found to contain 0.01 per cent of nitrogen. The first media prepared using this alkaline extract were made up thus:

[3]Bull. 95, Georgia Expt. Stat.

X. "Artificial Humus" Agar, (A),
Same as I. except that 20 c.c. of the extract of humus was used instead of the peptone.

XI. "Artificial Humus" Agar, (B).
Same as I. except that 10 c.c. of the extract of humus was employed in place of the peptone.

These eleven media were used in the tests of one soil, a typical Marshall loam which was obtained from the field with the usual precautions against contamination.

One hundred gms. were shaken for five minutes with 200 c.c. of sterile, distilled water and then dilutions of 1-2,000; 1-20,000 and 1-200,000 were prepared using sterile pipettes for the transfers and sterile distilled water for the diluting material.

One c.c. portions of the 1-20,000 and 1-200,000 dilutions were plated in duplicate with the eleven media just described.

The plates in this and subsequent series were incubated at about 25 degrees C. for three days and no difficulty was experienced from the overgrowth of molds.

Table I gives the results obtained with the media employed the counts on the duplicate plates being recorded. The moisture was determined in the soil used and the results are expressed as usual, on the air-dry basis.

TABLE I.

BACTERIA PER GRAM OF AIR-DRY SOIL

Medium	A.	B.	Average
I. Modified Synthetic Agar	5,546,000	5,411,000	5,478,000
II. Urea Agar	6,085,000	5,889,000	5,987,000
III. Asparagin Agar	4,662,000	4,294,000	4,478,000
IV. Casein Agar (A).	7,263,000	6,814,000	7,038,000
V. Albumen Agar, (A).	6,895,000	6,576,000	6,735,000
VI. Albumen Agar, (B).	3,435,000	3,385,000	3,410,000
VII. Temple's Peptone Agar	5,938,000	5,644,000	5,791,000
VIII. Temple's Soil Extract Agar	588,000	441,000	514,000
IX. Temple's Soil Extract Agar, Modified	4,368,000	4,171,000	4,269,000
X. "Artificial Humus" Agar, (A).	196,000	186,000	191,000
XI. "Artificial Humus" Agar, (B).	215,000	225,000	220,000

Looking over this table we note that the urea agar yielded somewhat larger numbers than the modified synthetic agar, the asparagin agar fewer numbers, while the casein agar, (A), and the albumen agar, (A), both gave considerably larger counts. Albumen agar, (B), on the other hand gave a smaller count than the modified synthetic agar. Evidently the albumen which was used in larger quantity instead of the dextrose failed to offer similar opportunities for growth as those afforded by the dextrose. Temple's peptone agar permitted the development of a somewhat larger number of organisms than the modified synthetic agar, but less than the urea, casein, or albumen, (A), agars. The soil extract agar showed only a few colonies developing.

Just what is lacking in this case is hard to say but it would seem that the soil extract failed to supply some essential constituent which is not contained in the peptone. The modification of this soil extract agar which was made up with the same soil extract but received only a small amount of peptone showed much greater counts but smaller than the modified synthetic agar. This would indicate that the large amount of peptone in the previous medium restricted the development of many organisms and that in both cases either the soil extract did not supply some necessary ingredient or added some injurious materials. Both artificial humus agars yielded very small counts due either to the lack of some necessary constituent or as was deemed possible, to their reaction, the humus extract making the agar slightly alkaline.

SERIES 2.

Eliminating those media which yielded smaller counts than the modified synthetic agar, Series 1 was repeated, using a soil obtained from a different source. The results obtained with this soil are given in table II:

TABLE II.

BACTERIA PER GRAM OF AIR- RY SOIL

Medium	A.	B.	Average
I. Modified Synthetic Agar	5,000,000	5,400,000	5,200,000
II. Urea Agar	5,650,000	5,800,000	5,725,000
IV. Casein Agar, (A).	7,250,000	7,475,000	7,362,000
V. Albumen Agar, (A).	8,050,000	7,500,000	7,775,000
VII. Temple's Peptone Agar	5,000,000	5,450,000	5,225,000

The same differences between the various media which appeared in Series 1 are shown here. Again with another soil, the urea, casein, albumen, and Temple's peptone agars yielded larger counts than the modified synthetic agar. There was only one variation in the ranking of the various media with respect to the number of organisms developing thereon and that was in the case of the albumen agar. While in Series 1 the casein agar seemed somewhat better, in this series the albumen agar gave the largest count, casein ranked second, then the urea agar, and finally Temple's peptone which showed only a slight gain over the modified synthetic agar.

From a consideration of these two series it appears that the casein and albumen agars containing as they do complex nitrogenous compounds present opportunity for the development of many organisms which do not appear when peptone is used as in the modified synthetic agar, and Temple's peptone agar.

SERIES 3.

Further tests of these media seemed desirable and it was deemed advisable also to attempt to make the artificial humus agar more satisfactory by regulating the reaction more carefully. To this end the following were prepared:

XII. "Artificial Humus" Agar, (C).
Same as I. except that 6.0 gms of K_2HPO_4 were used and 20 c.c. of the extract of humus replaced the peptone, the medium having a reaction of 0.5% acid.

XIII. "Artificial Humus" Agar, (D).
Same as I. except that 3.0 gms of K_2HPO_4 were used and 10 c.c. of the humus extract replaced the peptone. The reaction was 0.5% acid.

XIV. "Artificial Humus" Agar, (E).
Same as I. except that 3.0 gms. of the K_2HPO_4 were used and 10 c.c. of the humus extract replaced the peptone. The dextrose was omitted. The reaction here was 0.5% acid.

A sample of a different soil from those used in Series 1 and 2 was obtained and plates were made in triplicate in the usual way. The agreement of the triplicate determinations was very good and the differences brought out by the averages were quite distinct.

In table III will be found the results obtained with the five media employed in series 1 and 2 and with three additional media just described.

TABLE III.

BACTERIA PER GRAM OF AIR-DRY SOIL

Medium.	A.	B.	C.	Average
I. Modified Synthetic Agar	4,705,000	4,941,000	5,011,000	4,866,000
II. Urea Agar	5,741,000	5,555,000	5,411,000	5,569,000
IV. Casein Agar, (A).	6,447,000	6,164,000	5,647,000	6,086,000
V. Albumen Agar, (A).	7,058,000	7,270,000	7,011,000	7,113,000
VII. Temple's Peptone Agar	5,270,000	5,223,000	4,705,000	5,066,000
XII. "Artificial Humus" Agar, (C)	110,000	112,000	108,000	110,000
XIII. "Artificial Humus" Agar, (D)	4,000,000	3,811,000	3,670,000	3,827,000
XIV. "Artificial Humus" Agar, (E)	4,894,000	5,011,000	5,176,000	5,027,000

The relations between the modified synthetic agar, casein, urea, albumen and Temple's peptone agar which were shown in Series 2 were checked here. Again the modified synthetic agar showed fewer colonies than the other media, Temple's peptone agar second, then the urea agar, then the casein, and finally the albumen agar giving the largest count.

The artificial humus agar, (C), allowed of but a very small development of organisms, possibly due to the large amount of potassium phosphate introduced to make the reaction of the medium acid. The artificial humus agar, (D), gave a smaller count than the modified synthetic agar but many times as large

as the previously mentioned humus agar. The third humus agar, (E), permitted a larger number of organisms to develop than the other humus agars and the modified synthetic agar but fewer than the urea, casein, albumen, or Temple's peptone agars. Evidently the omission of the dextrose where the humus extract was employed favored the growth of some species and the utilization of both the nitrogenous and carbonaceous materials in the humus are clearly shown by the large counts obtained. The acid reaction of these agars permitted of greater growth than occurred in the case of the similar media previously tested which were of an alkaline reaction.

SERIES 4.

In this series a further check of previous results was carried out, using also three other media, modification of some previously employed.

XV. Casein Agar, (B).
Same as I. except that 0.05 gm. casein replaced the peptone.

XVI. Alubumen Agar, (C).
Same as I. except that 0.05 gm. of albumen was used instead of the peptone.

XVII. "Artificial Humus" Agar, (F).
Same as I. except that no dextrose was added and 10 c.c. of the humus extract, neutralized with normal HCl. was used instead of the peptone.

The first two of these media were very similar to two used in the three previous series, that is IV and V. These contained 0.10 gm. of casein and albumen respectively and it was thought advisable to determine if a smaller amount would yield better or even as good results.

The artificial humus agar was prepared using a solution of the humus neutralized with normal hydrochloric acid to eliminate the excess of potassium phosphate required when that material was employed to adjust the reaction to 0.5 per cent acid.

Furthermore no dextrose was supplied on the evidence obtained that there was enough carbonaceous material added in the humus extract.

The results obtained in this series are given in table IV a sample of still a fourth soil being employed and triplicate plates prepared as previously.

Again with the soil employed in this series, the same relations between the modified synthetic agar and the urea, casein, albumen and Temple's peptone agars were found to exist, the albumen as before giving the greatest count.

The artificial humus agar, (E), again gave about the same numbers as the modified synthetic agar, being slightly less in this

TABLE IV.

BACTERIA PER GRAM OF AIR-DRY SIOL

Medium.	A.	B.	C.	Average
I. Modified Synthetic Agar	4,666,000	4,844,000	4,555,000	4,688,000
II. Urea Agar	4,833,000	5,066,000	5,244,000	5,081,000
IV. Casein Agar, (A).	5,733,000	6,000,000	5,822,000	5,851,000
V. Albumen Agar, (A).	6,400,000	6,444,000	6,550,000	6,466,000
VII. Temple's Peptone Agar	4,622,000	4,666,000	4,844,000	4,710,000
XIV. "Artificial Humus" Agar, (E).	4,888,000	4,622,000	4,444,000	4,651,000
XV. Casein Agar, (B).	5,377,000	5,644,000	5,511,000	5,510,000
XVI. Albumen Agar, (C).	5,222,000	5,333,000	5,511,000	5,355,000
XVII. "Artificial Humus" Agar, (F).	5,666,000	5,777,000	4,444,000*	5,721,000

*Not included in the average.

instance. The humus agar, (F), however, gave much larger counts than the modified synthetic agar or the urea agar, but slightly less than the casein and albumen agars.

The medium in which the humus extract neutralized with hydrochloric acid was used was thus shown to be much superior to those in which large amounts of potassium phosphate were employed to give an acid reaction.

The casein agar, (B), and the albumen agar, (C), gave fewer numbers than the corresponding media, casein agar, (A), and albumen agar, (A), which contained 0.10 gm. of casein and albumen per liter of medium respectively. This amount evidently offered opportunity for greater development than the smaller quantities. In this table also we note good agreement among triplicates, in only one case was a count recorded which could not be included in the average.

SERIES 5.

In this series only one additional medium was tested. This was another modification of the artificial humus agar, using a larger amount of the humus extract.

XVIII. "Artificial Humus" Agar, (G).

Same as I. except that no dextrose was used and 25 c.c. of humus extract neutralized with normal HCl. replaced the peptone.

Temple's peptone agar was eliminated as the results had shown no striking advantage for it over the modified synthetic agar and the artificial humus agar, (E), was also omitted from further tests. The results obtained with the soil used in this series appear in table V.

Glancing over this table we find that the albumen agar gave again the largest count of the first four media. Comparing the results obtained with media XV and XVI with those secured using media IV and V just as was observed in Series 4, the

TABLE V.

BACTERIA PER GRAM OF AIR-DRY SOIL

Medium.	A.	B.	C.	Average
I. Modified Synthetic Agar	4,363,000	4,545,000	4,772,000	4,560,000
II. Urea Agar	5,090,000	5,000,000	4,909,000	4,999,000
V. Albumen Agar, (A).	6,181,000	5,954,000	5,863,000	5,999,000
IV. Casein Agar, (A).	5,681,000	5,818,000	5,522,000	5,673,000
XV. Casein Agar, (B).	5,272,000	5,182,000	5,318,000	5,257,000
XVI. Albumen Agar, (C).	5,590,000	5,681,000	5,727,000	5,666,000
XVII. "Artificial Humus" Agar, (F)	5,863,000	5,681,000	5,590,000	5,711,000
XVIII. "Artificial Humus" Agar, (G)	6,295,000	6,113,000	5,901,000	5,103,000

latter gave the larger counts, due evidently to the larger amounts of casein and albumen present.

The artificial humus agar, (F), gave a larger count here than all the media except the albumen, (A), agar, while in the previous series it was surpassed by the casein agar, (A), also.

The artificial humus agar, (G), gave still larger counts than the humus agar, (F), and slightly larger even than the albumen agar, (A). The larger amount of humus supplied in this last humus agar, (G), probably because of the greater food supply present, offered opportunity for the development of more organisms.

SERIES 6.

In this series three modifications of the albumen and casein agars using larger amounts of these materials were tested.

Further evidence was also sought on the relative advantages of the media which up to this point had indicated the greatest value, namely the casein agar, (A), the albumen agar, (A), and the artificial humus agar, (G).

The new media included here were made up thus:

XIX. Albumen Agar, (D).
Same as I. except that 0.5 gm. of albumen replaced the peptone.

XX. Casein Agar, (C).
Same as I. except that 0.5 gm. of casein was used instead of the peptone.

XXI. Albumen Agar, (E).
Same as I. except that 1.0 gm. of albumen was added instead of peptone.

The results obtained with these three media together with those secured with the media already tested, may be found in table VI.

It is evident again from a study of this table that the albumen agar, (A), gives opportunity for the development of more organisms than the modified synthetic agar, and slightly more than the casein agar, (A). Its superiority over the albumen agar, (C), containing a smaller amount of albumen is also clearly

TABLE VI.

BACTERIA PER GRAM OF AIR-DRY SOIL

Medium.	A.	B.	C.	Average
I. Modified Synthetic Agar	2,913,000	3,304,000	3,043,000	3,086,000
IV. Casein Agar, (A).	3,826,000	3,934,000	4,076,000	3,945,000
V. Albumen Agar, (A).	4,086,000	4,260,000	4,130,000	4,158,000
XVI. Albumen Agar, (C).	3,173,000	3,391,000	3,521,000	3,361,000
XVIII. "Artificial Humus" Agar, (G).	3,739,000	3,826,000	3,913,000	3,826,000
XIX. Albumen Agar, (D).	3,869,000	3,956,000	3,652,000	3,825,000
XX. Casein Agar, (C).	3,695,000	3,521,000	3,804,000	3,673,000
XXI. Albumen Agar, (E).	2,956,000	3,086,000	3,260,000	3,034,000

shown. The medium, XIX, albumen agar, (D), containing 0.5 gm. of albumen per liter gave a smaller count than the albumen agar (A), and XXI, albumen agar, (E), with 1.0 gm. of albumen agar per liter yielded a still smaller count. Evidently the optimum amount of albumen in the medium is 0.10 gm. per liter. The casein agar, (C), containing 0.5 gm. of casein per liter yielded smaller results than the casein agar, (A), so that in this case also the optimum amount of casein in the medium seems to be 0.10 gm. per liter.

The artificial humus agar, (G), which in the previous series gave a slightly larger number than the albumen agar, (A), in this case proved slightly inferior to this agar and also to the casein agar, (A). The differences were very slight in both cases so that we are justified in concluding that the artificial humus agar, (G), was not superior to the albumen agar, (A), at least and possibly not to the casein agar, (A).

Considering the results of these experiments as a whole we find that in most instances the modifications of the modified synthetic agar supplying more complex nitrogenous materials in place of the peptone permitted of the development of more organisms. Particularly was this the case when casein and albumen replaced the peptone, and when a neutralized extract of humus was substituted for both the peptone and the dextrose.

The media in which the peptone was replaced by simpler nitrogenous compounds as asparagin and urea, in one case proved inferior and in the other very slightly superior to the modified synthetic agar.

The soil extract agars which were tested proved quite inferior to the modified synthetic agar. This fact in addition to the great objection to them because the results obtained in one locality cannot be checked at any other place on account of the variability of soil extracts, discourages their use.

The experiments with variations of the albumen and casein agars showed that the optimum amounts of these materials were 0.10 gm. per liter. In the case of both these media no

difficulty whatever with "spreaders" was encountered. These sometimes interfere when the modified synthetic agar is used. The only objection to the use of casein and albumen is the fact of their coagulation when heated, but, as has been stated, this is not serious as the coagulum is readily broken up by shaking the tubes vigorously before pouring the material on the inoculum in the Petri dishes and the slight cloudiness of the medium offers no difficulty in making accurate counts.

With regard to the relative advantages of the casein and albumen media, in one case we find the casein gave the largest count but with all the other soils the albumen seemed to be the best, so that the preponderance of evidence is in favor of the latter medium.

The humus extract agar, (G), proved about equal to the albumen agar in value, in one case slightly surpassing it and with another soil proving somewhat inferior. The difficulties attendant upon the preparation of this medium are much greater than in the case of the albumen agar and it is slightly less constant in composition and hence it can hardly be considered preferable to the albumen agar.

This albumen agar has been tested quite extensively in connection with other work since these experiments were carried on and it has consistently shown much larger numbers than the modified synthetic agar and no difficulty has been experienced in its use.

CONCLUSIONS

These experiments, as a whole, show, therefore, that for quantitative estimations of soil bacteria:

1. Albumen agar of the same composition as modified synthetic agar except that the peptone is replaced by 0.10 gm. of albumen per liter permits the development of much larger numbers of bacteria than the modified synthetic agar or any other medium tested, except an artificial humus agar.

2. There are no serious difficulties attendant upon its use as has been conclusively shown in numerous more recent experiments.

3. Casein agar, similar to the albumen agar, containing 0.10 gm. of casein per liter as the nitrogen source shows also a much larger number of organisms developing on it than the modified synthetic agar, but slightly smaller than the albumen agar.

4. Artificial humus agar containing 25 c.c. per liter of a neutralized sodium hydroxide extract of humus prepared from oats straw, and otherwise of the same composition as the modi-

fied synthetic agar except that no dextrose is employed, yielded practically the same counts as the albumen agar.

5. The difficulties in the preparation of this material and the fact that it is not superior to the albumen agar lead naturally to the recommendation of the latter medium.

6. Media prepared from soil extracts permitted fewer organisms to develop than the modified synthetic agar, due either to the lack of some essential food constituent or to the introduction of some substance injurious to certain species.