STUDIES ON SOIL *ACTINOMYCES*. PART III. STANDARDISATION OF A PLATE METHOD OF COUNTING SOIL *ACTINOMYCES*.

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It was shown in the previous communication (*J. Indian Inst. Sci.*, 1929, **12 A**, 57) that in the normal soil *Actinomyces* are found almost exclusively in the form of conidia, and that, when they occur vegetatively, as they do in some rare cases, their mycelia are largely present on undecomposed plant residues and similar forms of organic matter which are generally removed during sampling, and not on the soil itself. Since the conidia resemble many of the commoner forms of bacteria in shape and size, no direct method of counting them, stained or otherwise, will be feasible. The plate method alone can provide some information regarding the numbers and distribution of *Actinomyces* in soil.

The plate method will not, however, be satisfactory unless it fulfils the following conditions:—(I) The count medium should (*a*) be composed of materials well-defined in composition and character, (*b*) be readily reproducible, (*c*) contain minimal quantities of essential nutrients and bring out the largest numbers without encouraging any abnormal vegetative growth, (*d*) bring into relief the cultural characteristics distinguishing different species of *Actinomyces* not only from other microflora, but also from each other, and (*e*) discourage growth of other organisms, particularly those of spreading bacteria and fungi. (II) Details relating to the making of plates should be readily applicable not only to all types and conditions of soils but also to different species of *Actinomyces* present in them. (III) Parallel plates should provide counts which are (*a*) within limited range of variation from each other, and (*b*) distributed in a well-defined manner over the plates.

Though numerous media have been used by different workers, none of them has yet been tested for its fitness to be used as the standard medium for counting *Actinomyces*. The earlier workers used exclusively beef extract media solidified with agar or gelatin. Among the later workers Krainsky (*Zentr. Bakt.*, 1914, II, **41**, 649) found these unsatisfactory, preferring calcium malate agar and similar synthetic media. Conn (*J. Bact.*, 1916, **1**, 197) used an agar medium containing sodium asparaginate and glycerol. Waksman and Curtis (*Soil Sci.*, 1916, **1**, 99) noted that a medium containing albumen was efficient for

counting *Actinomyces* together with bacteria. As a result of applying such diverse media, counts obtained by different workers, though useful by themselves, were not comparable with each other.

The object of the present investigation was to develop a standard method for counting soil *Actinomyces* which would satisfy the conditions set forth already.

EXPERIMENTAL.

Preliminary trials with the commoner plate and culture media were carried out with a view to (*a*) determining the extent to which each could approach the ideal, and (*b*) obtaining a line for further improvement on the most promising among them. The media tried were: (1) Modified albumen agar (Waksman and Curtis, *loc. cit.*), (2) Glucose agar (Krainsky, *loc. cit.*), (3) Soil extract agar (Fischer: Waksman, *Principles of Soil Microbiology*, 1927, pp. 15, 16 and 291), (4) Casein agar (*ibid.*), (5) Nutrient agar with 1 per cent. glycerol, (6) Synthetic agar (Lipman and Brown, *ibid.*), (7) Malate, glycerol agar (Krainsky, *loc. cit.*), (8) Sodium asparaginate glycerol agar (Conn, *loc. cit.*), (9) Urea, ammonium nitrate agar (Cook, *Soil Sci.*, 1916, 1, 153), (10) Starch agar, (11) Citrate, glycerol agar (Conn, *N. Y. Agric. Exp. Sta. Tech. Bull*, 60, 1917) and (12) Czapek's agar which, with 10, is also specified in Waksman's book. Four specimens of soil representing the types obtained in different parts of India were used for the platings. They were surface samples (0–9″ depth) of (A) black cotton soil from Hyderabad, Deccan, (B) alkaline, wheat-growing soil from Gujranwala District, Punjab, (C) peaty soil from Travancore, South India and (D) low-lying paddy land from Dacca, Bengal.

The technique adopted for plating in the preliminary and most of the subsequent trials was as follows:—After being air-dried, powdered and passed through the millimeter mesh sieve, 10 gms. of the soil were shaken vigorously for 5 minutes with 100 c.c. of sterile tap-water in a stoppered flask, 10 c.c. of the suspension removed before settling and transferred to a second flask containing 90 c.c. of sterile tap-water. The second suspension was shaken for one minute and 10 c.c. transferred to a third flask containing 90 c.c. of sterile tap-water. After being shaken again for one minute, 1 c.c. of this third suspension was transferred to 90 c.c. of sterile tap-water, and 1 c.c. portions of this fourth suspension used with frequent shaking for the platings, which were carried out in quadruplicate. Incubations were at 25–30°, and the final counts taken at the end of a fortnight. In the preliminary trials observations and counts were made once in two days to compare the relative growths of the different forms of microflora appearing on them.

Before discussing the results, it may be mentioned that most of the above-mentioned media were slightly acid, though in a few cases the reactions were adjusted to be neutral. With the exception of medium 3 all contained at least 1 per cent. of glucose, sucrose, or glycerol as the main carbon nutrient. Media 1, 2, 6, 7, 9 and 11 contained less than 0·05 per cent. of nitrogenous nutrients which were egg albumen, asparagine, peptone, ammonium chloride (7 and 11), urea and ammonium nitrate respectively: their P_H as measured colorimetrically (Medalia, *J. Bact.*, 1920, **6**, 441) was also varied, being 6·4, 5·3, 5·4 and 7·0 (7, 9 and 11) respectively. The nitrogen content of 3 was very low and of indeterminate composition; the P_H of that medium was 6·2. Media 4, 8, 10 and 12 contained between 0·1 and 0·2 per cent. of casein, dihydrogen ammonium phosphate, ammonium sulphate and sodium nitrate respectively; their P_H was 6·2, 6·8, 7·0 and 6·4 respectively. Medium 5 was richest in nitrogen and contained 1 per cent. peptone. The mineral contents of the different media did not vary appreciably from each other.

In the present and the subsequent trials, *Actinomyces* appearing on the plates were distinguished from other organisms by the following cultural characteristics:—(I) Formation of (*a*) round or very nearly round colonies, which spread only slowly, (*b*) aerial mycelia which under favourable conditions rose above the medium-surface and were composed of very fragile hyphæ, disrupted by gentle prodding with the needle, (*c*) concentric 'fairy-rings' composed almost exclusively of reproductive hyphæ and conidia, (*d*) the characteristic earthy odour detected in most cases after even the merest touch of the colony with a sterile needle, (*e*) variegated colours formed by either or both substrate growths and aerial hyphæ. (II) Microscopic appearance of the colony edges which, being composed of thin, close-packed hyphæ, distinguished *Actinomyces* readily from all other forms of micro-organisms. Repeated trials showed that even when none of the characteristics mentioned under I were exhibited, *Actinomyces* never failed to present the characteristic edge appearance (II) at any stage during growth.

The observations showed that although on most of the plates *Actinomyces* began appearing before the end of one week, they could not, in the earlier stages, be readily distinguished from bacteria except by microscopic examination. Growths on medium 10 were, however, prominent from the beginning and exhibited all the characteristics mentioned already. The total numbers of micro-organisms including *Actinomyces*, counted at two-day intervals, presented a confusing mass of data. From the three following typical sets of counts (Table I) taken after one-week's incubation for two soils, it will appear that,

TABLE I.

Medium No.	Colonies per gm. of soil (millions)	
	Soils	
	A	C
2	2·0	1·1
4	3·7	3·9
11	1·9	6·2

depending on the medium used, Soil C contained numbers which were (*a*) less than, (*b*) equal to, and (*c*) much greater than those present in Soil A. All the data were, therefore, discarded.

It was also observed that the total numbers did not increase steadily with appearance of new colonies from time to time; the counts rose and fell spasmodically. Microscopic examinations of circlets locating colonies that had been previously observed showed that many did not contain even traces of the original growths, thereby indicating that the older colonies steadily disappeared while new ones came up. The study of conditions leading to such disappearances will form the subject of a later communication.

When the final counts were made, it was observed that media 4, 5 and 12 encouraged growths of numerous fungi and bacteria which spread rapidly and almost completely covered the *Actinomyces* that appeared. No more than one or two poorly formed colonies of *Actinomyces* appeared on 3 which, obviously, lacked the minimal requirements of the organisms. Media 7, 8 and 11 brought out large numbers of spreading bacteria, but comparatively few *Actinomyces*, which made no satisfactory growth.

Media 1, 2, 6 and 9 carried fewer bacteria, but the *Actinomyces* showed no marked cultural characteristics differentiating them readily from other organisms. Medium 10 proved to be the most satisfactory and brought out a fairly large number of colonies with all their cultural characteristics: bacteria and fungi made very poor growths, and at no time interfered with the development of *Actinomyces*. The final counts (Table II) of *Actinomyces* taken at the end of the fortnight were rather low, indicating thereby that the soils were not comparable with those for which some previous workers (e.g., Conn, *loc. cit.*) using the same media obtained higher counts.

TABLE II.

Medium	*Actinomyces* per gm. of soil (100,000's)			
	Soils			
	A	B	C	D
1	9	11	8	10
2	10	8	4	5
3	*	*	*	*
4	16	10	9	14
5	*	*	*	*
6	5	10	7	9
7	7	7	8	6
8	7	7	8	6
9	7	8	10	7
10	12	15	13	7
11	8	14	11	5
12	7	8	6	9

The starch medium, in spite of its advantages, was difficult to work with, because it contained large amounts of (*a*) potato starch which made it sticky and (*b*) calcium carbonate which rendered it opaque.

Effect of altering the proportions of minerals and nitrogen in the starch medium.—To determine whether (1) the carbonate can be replaced by a soluble salt of calcium and (2) the quantities of nitrogen and minerals can be reduced to a minimum, platings were made using a medium (II) composed (in grams) of starch (potato), 10; HK_2PO_4, 0·5 $(NH_4)_2SO_4$, 0·2; $MgSO_4 7H_2O$, 0.2; $CaCl_2$, 0·1; $Fe_2(SO_4)_3$ a trace, in distilled water, 1,000 c.c. with washed agar, 20 grams, the P_H being adjusted to 7·2. The counts, as compared with those on the original starch medium (1), are shown below.

The deviations being alternately in favour of media I and II, it may be inferred that the change in composition had not altered significantly the counts of *Actinomyces*. Medium II was the clearer, but suffered more from spread of fungi.

* Practically no colonies, or uncountable owing to overgrowth of fungi.

TABLE III.

Starch medium	*Actinomyces* per gm. of soil (100,000's)			
	Soils			
	A	B	C	D
I	12	15	13	7
II	14	14	12	10

Varying the source of nitrogen.—To determine whether (1) starch was superior as a source of carbon to glucose which had been used by several of the earlier workers and (2) the use of other nitrogenous compounds in place of ammonium sulphate used in the previous trials could bring out larger numbers of *Actinomyces* and check more effectively the spread of fungi, platings were carried out with media containing the same proportions of minerals as II, but differing from it in containing other nitrogen compounds in place of ammonium sulphate together with 1 per cent. each of glucose (Series III) and potato starch (Series IV) respectively. Nitrogen was supplied in the forms of asparagine, 0·30 gm. (IIIa and IVa); ammonium chloride, 0·17 gm. (IIIb and IVb); sodium nitrate, 0·30 gm. (IIIc and IVc) and urea, 0·10 gm. (IIId and IVd) respectively added to every litre of medium. Control experiments were also carried out using a medium containing only starch and minerals. Soil B which gave consistent numbers in previous trials was used for plating. The counts (Table IV) showed that, although series IV did not give significantly better counts than series III, *Actinomyces* appearing on the former made very much better growths and were almost entirely free from

TABLE IV.

Actinomyces per gm. of soil (100,000's)									
Glucose					Starch				
III (control)	IIIa	IIIb	IIIc	IIId	IV (control)	IVa	IVb	IVc	IVd
9	10	8	10	9	11	13	12	14	12

spreading bacteria and fungi. The colonies appearing on control plates were poorly formed, indicating that the nitrogen requirements of

Actinomyces, though small, should nevertheless be satisfied. The plates containing asparagine were the cleanest: those containing urea were marred by spread of fungi.

Effect of reducing the quantities of nitrogen and starch.—To ascertain whether (1) the supply of nitrogen can be further reduced, (2) mixtures of nitrogenous compounds will prove more effective than single substances and (3) the quantity of starch can be lowered, trials were carried out using media (Va–Vd) containing the same amounts of minerals as in the previous trials, together with asparagine, 0·05 gm. and (Va) sodium nitrate, 0·05 gm. and potato starch, 10 gms., (Vb) ammonium chloride, 0·05 gm. and potato starch, 10 gms., (Vc) sodium nitrate, 0·05 gm. and potato starch, 2 gms., and (Vd) ammonium chloride, 0·05 gm. and potato starch, 2 gms.—per litre of medium in each case.

It was observed that reduction in the quantity of starch rendered the media easier to handle and markedly increased the counts (Table V).

TABLE V.

Soil	*Actinomyces* per gm. of soil (100,000's)			
	Va	Vb	Vc	Vd
B	15	12	22	16

It was also noticed that plates containing asparagine and nitrate (Vc) not only carried the largest numbers of colonies but were also the cleanest. Media containing ammonium chloride encouraged growth of fungi.

Starches from different sources.—Potato starch which was used in the previous trials was not satisfactory because it (1) rendered the medium cloudy, and (2) was not uniform in quality. To ascertain whether more satisfactory counts can be obtained by using starches from other sources, trials were carried out with media (VIa–VId), identical with Vc except that rice (VIa), maize (VIb), wheat (VIc) and soluble (B.D.H., A.R.) starches (VId) were used in place of that from potato. Soils A and B were used for the platings. The counts are given in Table VI.

TABLE VI.

Soil	*Actinomyces* per gm. of soil (100,000's)				
	Vc	VIa	VIb	VIc	VId
A	29	11	21	19	31
B	22	9	18	14	23

It was observed that growths of *Actinomyces* on rice-starch were poor, while those on maize and wheat-starch were largely overgrown with fungi. The soluble starch medium, though not superior to that of potato starch in the numbers, was otherwise more satisfactory because of its clarity and greater efficiency in checking spread of fungi. Soluble starch possessed also the advantage of being a standard product of reproducible quality.

Effect of changing the quantity of soluble starch.—To ascertain whether the proportion of soluble starch can be altered with advantage, trials were carried out with media identical with VId but containing different amounts of soluble starch. They were, (VIIa) only minerals and nitrogen, without any starch, as control: VIIb–VIIe containing 0·05, 0·1, 0·2, and 0·3 per cent. respectively of soluble starch: and VIIf only 0·2 per cent. starch, without minerals or nitrogen, as control. Soils A, B, C and D were used. The counts are given in Table VII.

TABLE VII.

Medium	*Actinomyces* per gm. of soil (100,000's)			
	Soils			
VII	A	B	C	D
a	10	7	6	3
b	7	7	...	3
c	9	8	5	4
d	28	22	25	10
e	16	36	17	5
f	3	10	9	2

It was observed that colonies appearing on media VIIa, VIIb, VIIc and VIIf were not only numerically small, but also poorly formed, indicating that the media did not supply the minimal requirements of the organisms for making satisfactory growth. Counts on VIIe were less consistent than those on VIId.

It is interesting to note that there was much greater response from *Actinomyces* to (1) starch from potato rather than to that from any of the grains, (2) one particular concentration of soluble starch than to others. A study of the causes leading to such observation is being made and will form the subject of a later communication.

Replacing soluble starch by other carbohydrates.—Platings were made in media identical with VIId but containing 0·2 per cent. each of maltose (VIIIa), lactose (VIIIb), dextrin (VIIIc), inulin (VIIId), glycogen (VIIIe), fructose (VIIIf) and xylose (VIIIg) in place of soluble starch.

The poor counts that were obtained (Table VIII), together with the observation that the colonies were meagrely formed, showed that none of the carbohydrates was favourable to the development of

TABLE VIII.

Medium VIII	*Actinomyces* per gram of soil (100,000's)			
	Soils			
	A	B	C	D
a	10·8	3·2	*	1·2
b	10·2	6·4	9·6	4·8
c	8·2	10·4	*	1·4
d	4·2	3·4	*	5·2
e	10·2	7·8	1·4	9·4
f	*	2·2	*	1·8
g	*	8·2	*	1·4

* No colonies, or completely overgrown with fungi.

Actinomyces. Profuse growths of bacteria and fungi occurring on the plates showed, on the other hand, that the media were suited only to such organisms.

The foregoing data confirm the previous observation (*vide* Table I) that counts on different media though for the same soils did not follow any order. They suggested that formation of colonies on count media were determined not only by the numbers of living cells present in the soils but also by the individual response of the different organisms to the nutrients provided by the media. The latter property in the case of soil organisms would depend on the previous conditions such as manuring and cropping of the soils concerned.

Effect of increasing the asparagine and nitrate.—Platings were made on media identical with VIId but containing asparagine and nitrate (IXa), 0·1 gm. each; (IXb) 0·2 gm. each; (IXc) 0·5 gm. each respectively per litre of medium. The counts (Table IX) showed that

TABLE IX.

Soil	*Actinomyces* per gm. of soil (100,000's)			
	VIId	IXa	IXb	IXc
B	22	14	13	8

in addition to giving lower counts, the media containing larger amounts of nitrogen encouraged growths of spreading bacteria and fungi. To suppress spreading colonies it would therefore be necessary not only to choose the proper forms of carbon and nitrogen, but also to maintain their quantities at the minimum.

Altering the mineral composition of the medium.—To find whether (1) all the mineral and nitrogen compounds used in the previous trials were essential, (2) some of the ingredients can be replaced advantageously by allied compounds, and (3) addition of certain new substances can lead to marked alteration in the number of colonies, trials were conducted with media which were the same as VII*d*, except in the following respects:—Xa, without dipotassium phosphate; Xb, without asparagine; Xc, without sodium nitrate; Xd, without ferric sulphate; Xe, without magnesium sulphate; Xf, without calcium chloride; Xg, with potassium nitrate in place of the sodium salt; Xh, with disodium phosphate in place of the dipotassium salt; Xi, together with 0·01 per cent. zinc chloride; Xj, together with 0·01 per cent. aluminium acetate. P_H was adjusted to 7·4 in all cases.

The zinc salt was added to determine whether it had a stimulating effect similar to that it has generally on fungi. Since free aluminium is known to be present in many acid soils which do not encourage the growth of commoner forms of *Actinomyces*, the aluminium salt was

added to determine whether it had any inhibitory action. The platings were carried out with soils A, B, C and D. The counts (Table X) showed that none of the media was as satisfactory as VIId.

TABLE X.

Medium X	*Actinomyces* per gram of soil (100,000's)			
	Soils			
	A	B	C	D
a	6·4	16·4	15·2	6·2
b	13·6	15·8	13·2	8·8
c	14·8	18·8	14·6	5·4
d	15·0	19·4	9·8	6·6
e	6·8	16·6	17·0	7·8
f	10·4	17·6	13·2	13·4
g	5·8	17·8	12·6	5·8
h	13·8	20·0	18·2	4·4
i	8·2	17 0	18·4	1·8
j	3·8	14·8	12·2	9·8

It is interesting to observe that the counts for soils B and D were affected only slightly by alteration in mineral-supply and those for C to a slightly greater extent, while those for A were considerably lowered in every case. The varied response given by *Actinomyces* from different soils suggested that their previous conditions should have determined such behaviour. It is thus possible that the organisms from soils B and D had sufficient reserves of the essential mineral nutrients while those from A had not, so that when plated on media lacking one or the other of the salts, the organisms from B and D did not miss them while those from A did.

The counts on media Xa to Xj show the importance of every one of the components of medium VIId in helping to bring out a satisfactory number of colonies. The data for Xg and Xh show that sodium and potassium could not advantageously replace each other: those for Xi and Xj show that addition of zinc chloride and aluminium acetate led to no improvement in the counts.

It was also observed that the plates unsatisfactory with regard to counts of *Actinomyces*, generally carried larger numbers of fungi and spreading bacteria than the normal ones.

Reaction of the count medium.—To determine the most favourable reaction, platings were made on media which had the same composition as VIId, with the P_H adjusted as follows:—XIa, 6·2; XIb, 6·6; XIc, 7·0; XId, 7·4; XIe, 7·8 and XIf, 8·2. Medalia's colorimetric standards (*loc. cit.*) were used. The counts given in Table XI showed that the best were obtained only when the media were slightly alkaline.

TABLE XI.

Soil	*Actinomyces* per gm. of soil (100,000's)					
	XIa	XIb	XIc	XId	XIe	XIf
A	18	21	27	30	29	21
B	13	17	24	25	26	26

Although XId and XIe gave almost identical counts, the colonies on the former were better formed than those on the latter. P_H 7·4 was inferred to be the most favourable.

It was observed that above P_H 8·0, the colonies dwindled in size, while below P_H 7·0, though the numbers decreased, the colonies were larger and better-formed than the normal ones. The observation suggested that (1) *Actinomyces* were not tolerant of more than slight alkalinity and (2) certain species throve better in acid, than neutral or alkaline conditions. *A. acidophilus* (*Soil Sci.*, 1928, **25**, 225) is probably one of these.

Preparation of the Standard Medium.

The foregoing trials having shown that the best counts of *Actinomyces* were obtained with medium VIId, attempts were made to standardise the conditions for its preparation. On mixing the components in different ways, it was observed that potassium phosphate led, on boiling, to precipitation of magnesium, calcium and iron which were removed on filtering. Previous addition of starch in solution, however, protected the salts from coagulation and preserved them in a finely divided state which passed the filter readily. The protective action was greatly increased by adding the necessary amount of molten agar along with starch.

The following method was found to be satisfactory for preparing the medium:—Standard soluble starch (2 gms.) was made into a uniform paste with about 25 c.c. of distilled water, the volume being increased to about 250 c.c. and the mixture autoclaved for 10 minutes at a pressure of 5 lbs. to gelatinise the starch and to bring it to even dispersion; 500 c.c. of filtered, 4 per cent. washed agar were melted at the same time and added to the hot starch solution with frequent stirring. Dipotassium phosphate (50 c.c. of a 1·0 per cent. solution) was then added slowly to the starch-agar mixture, with frequent stirring. The other ingredients of the medium (in grams) were $MgSO_4\ 7H_2O$, 0·2; $CaCl_2$, 0·05; $NaNO_3$, 0·05; asparagine, 0·05; and $Fe_2(SO_4)_3$ (1 drop 1 per cent. solution); these were made up in a separate mixture from stock 1 per cent. solutions and added. The whole was added in small instalments to hot agar-starch-phosphate mixture with stirring and raised to boiling before adjusting the reaction by Medalia's method (*loc. cit.*).

Standardisation of Plating Details.

The previous trials showed that air-dry specimens passed through the millimeter-mesh sieve provided uniform dispersion of the soil particles and give fairly uniform counts. But it was not clear whether the tap-water used was a really satisfactory disperse medium. Plating trials were therefore carried out comparing tap-water with the saline solution containing 5 gms. NaCl and 1 gm. $MgSO_4$, 7 H_2O in 1000 c.c. of distilled water recommended for plating soil bacteria (Thornton *Ann. Appl. Biol.*, 1922, **9**, 241). The counts (Table XII) were very

TABLE XII.

Disperse medium	*Actinomyces* per gm. of soil (100,000's)			
	Soil			
	A	B	C	D
Tap-water	32	25	29	11
Saline solution ...	33	23	26	13

nearly the same for both the disperse media showing that the conidial form in which the *Actinomyces* were mostly present was not sensitive to the osmotic conditions of the dispersing fluid.

It was observed that shaking soil suspensions by hand for definite periods did not necessarily lead to even dispersion of the soil particles

because (1) depending on the strength and temperament of the worker the suspensions were well or ill shaken during the same period, (2) shaking vigorously and steadily during 5 minutes for every case, particularly for numerous platings, was exacting, (3) whatever the time spent, if the shaking during the last stages was not vigorous and the suspensions were not removed before settling could occur, the counts proved to be unsatisfactory.

Trials were therefore conducted to determine whether (1) the time of shaking could be conveniently reduced, and (2) shaking vigorously and continuously for a fixed number of times would be more efficacious than an indefinite rate for a definite period. The suspensions were removed quickly before settling. The details of the experiments together with the counts obtained are given in Table XIII.

TABLE XIII.

	Mode of Shaking	*Actinomyces* per gm. of soil (100,000's)			
		Soils			
		A	B	C	D
I	Time—5 minutes ...	33·4	24·8	28·8	12·0
I	,, —3 ,, ...	31·8	25·6	26·4	10·6
I	,, —1 ,, ...	24·6	21·4	26·6	10·2
II	Number—200 ...	32·6	23·6	28·6	11·4
II	,, —150 ...	32·0	24·4	27·8	11·8
II	,, —100 ...	29·8	22·8	26·6	10·6

I. Slow with occasional stoppages: II. Vigorous non-stop.

The results showed that reduction in time of shaking to 3 minutes had not greatly affected the counts, while the further reduction to 1 minute had. Shaking vigorously for 200 or 150 times during about one minute gave results as good as those following less vigorous agitation during five minutes. It may be inferred that the vigour of shaking rather than the time occupied effected the dispersion of soil particles.

It was further observed that uniform counts could be obtained by shaking for only 100 times for the second and subsequent dilutions; not containing much solid matter, the suspensions became readily dispersed.

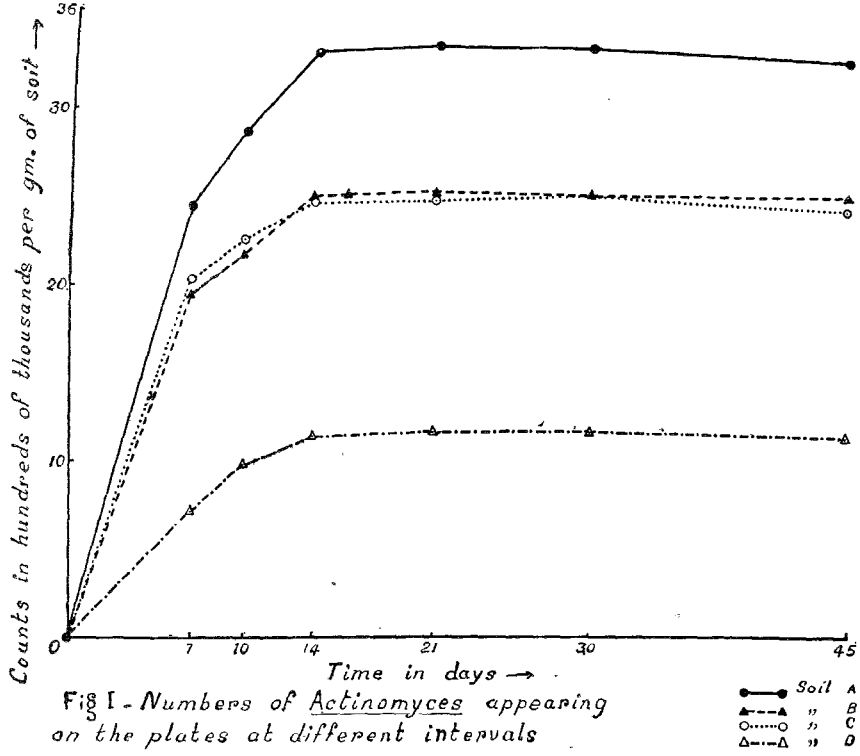

Fig I - Numbers of Actinomyces appearing on the plates at different intervals

Dilutions to be Employed.

Platings with soils from various localities showed that it was not always advantageous to adopt a dilution of 1 in 100,000. Counts (Table XIV) for soils from different parts of India and Ceylon showed that even 1 in 1,000 had to be employed in certain cases.

TABLE XIV.

Locality	Description	Crop raised	*Actinomyces* per gm. of soil (10,000's)
Hyderabad, Deccan	Black cotton ...	...	334
Gujranwala Dt., Punjab	Alkaline ...	Wheat ...	254
Travancore, S. India	Peaty ...	Paddy ...	234
Dacca, E. Bengal	Alluvial ...	Paddy ...	100
Bundelkhand, U. Provinces ...	...	...	216
Sholapur	Karl soil ...	...	95
Kandy, Ceylon	Reddish laterite ...	Tea ...	25
Mirphukas Farm, Sindh	Kalar soil ...	Fruit ...	68
Tellicherry, S. India	Red sandy loam ...	Coconut ...	4

The number of colonies of *Actinomyces* allowable on the plate depended to a large extent on the average size of the individual colonies and the other forms of microflora appearing at the same time, but under normal conditions 40 was found to be convenient. The best results were obtained by adjusting the dilution to give between 30 and 40 colonies per plate.

Period and Temperature of Incubation.

To ascertain whether incubation for a fortnight as in the previous trials was adequate, the platings were made on the standardised medium using soils A, B, C and D. The counts (Fig. 1) showed that (1) most of the colonies appeared simultaneously at the end of a week, and (2) subsequent increase in number was appreciable up to the end of a fortnight, but was insignificant in later stages.

Incubations at 37° showed that although colonies of *Actinomyces* appeared even at the end of about four days, they were more readily apt to be over-run by spreading organisms than at a lower temperature. It was also observed that variations of temperature between 25° and

30° did not appreciably affect the final counts. The same range of temperature is considered satisfactory for platings using the standard medium.

METHOD AND ACCURACY OF COUNTING.

Since *Actinomyces* were always prominent by their size and cultural characteristics and comparatively small in numbers, it was found possible, throughout this investigation, to count all the colonies that appeared on the plates. A similar method is recommended for use with the standard medium.

Consistency of individual counts.—To learn whether, under technically perfect conditions, use of the standard medium can give consistent counts, platings of soils from different parts of India and Ceylon were made. Dilutions of 1 in 100,000 were adopted in all the cases. The counts (Table XV) showed that they were fairly uniform, indicating

TABLE XV.

No.	Locality	Description	Counts on individual plates				
			I	II	III	IV	V
1	Jacobad, Punjab ...	Govt. wheat farm ...	30	32	28	32	...
2	Nasik, Bombay ...	Garden soil ...	32	32	34	28	31
3	Gaya, Bihar ...	Waterlogged ...	4	6	4	5	5
4	Chirakkal, Madras ...	Sandy loam, Paddy ...	35	46	40	38	42
5	Ratnagiri, Bombay ...	... ...	17	15	12	14	...
6	Jaffna, Ceylon ...	Sandy, Coconut ...	14	13	11	11	12

that under ideal conditions, which could not be attained experimentally because of the errors inherent to the dilution process, they should have been identical.

Because of the small numbers involved, errors will in certain cases be highly magnified in spite of the uniformity of individual counts. Thus the standard errors of the averages from different sets of counts represented as percentages (Table XVI) were, (1) 3·1; (2) 3·2; (3) 7·8; (4) 6·2; (5) 7·2; and (6) 4·8 respectively, showing that, although the individual counts of sets (*c*) and (*e*) were much more consistent than those of (*d*), the errors of their averages were greater than those of the latter because of the smallness of their counts. As suggested already, the adjustment of dilution for such soils to give between 30 and 40 colonies per plate will greatly minimise the error.

Distribution of counts from random samples.—Knowledge of the exact manner of distribution of the colonies on parallel plates is essential before adopting a technique in the study of problems relating to soil *Actinomyces*. Thus, if it be known that under technically perfect conditions the parallel counts will vary within a small range from their mean, then a count from a similar sample of soil which deviates beyond the recognised range will denote a significant difference in biological condition from the first sample. If, on the other hand, individual counts from the same sample vary considerably from each other, the technique of counting will be useless to detect small changes in numbers.

In their study of the accuracy of the plate method of counting bacteria, Fisher, Thornton and Mackenzie (*Ann. Appl. Biol.*, 1922, **9**, 325) observed that the colonies were distributed in the manner of samples of the Poisson series. Thus, if m be the known average of a number of bacterial counts, then the frequencies with which individual counts can bear values 0, 1, 2,....., n, ...will be given theoretically by the series :—

$$e^{-m}\left(1, m, \frac{m^2}{2!}, \frac{m^3}{3!}, \ldots\ldots, \frac{m^n}{n!}, \ldots\ldots\right)$$

Given the average, the mode of distribution of the individual counts can be calculated and used for checking the accuracy of those actually obtained by counting.

Since the plate method of counting *Actinomyces* was similar in principle to that adopted for bacteria, it was thought that in a like manner, the individual counts would be distributed in the manner of samples of the Poisson series.

To verify this, platings were made using a number of samples from one single specimen of soil. The Chirakkal soil (20 gms.) used in the previous trial was mixed with 200 c.c. of sterile saline and thirteen sets of dilutions of 1 in 100 were prepared from the suspension, using sterile tap-water in seven and sterile saline in the remaining six cases as disperse media. Further dilutions to 1 in 100,000 were then prepared in the manner already described using sterile tap-water and saline respectively. They were then plated on the standard agar medium using 5 plates for each set. From the counts obtained after a fortnight's incubation two statistics were calculated from each set of data. They were (1) the mean, $\bar{x}$ and (2) the variance, V which was calculated according to formula,

$$V = \frac{1}{n-1}. \quad S(x-\bar{x})^2$$

where n represented the number of plates and S $(x-\bar{x})^2$ the sum of squares of deviations of individual data from the mean. The object of the calculation was to ascertain whether the values of V approximated to those of $\bar{x}$ as should be the case with samples of the Poisson series. The figures thus calculated (Table XVI) showed that in every one of

TABLE XVI.

Tap Water		Saline Solution	
$\bar{x}$	V	$\bar{x}$	V
38·6	9·3	38·0	5·0
40·2	3·7	39·2	6·7
38·5	2·3	40·2	3·5
36·6	3·3	39·4	10·3
39·2	5·7	38·4	4·5
38·8	5·7	39·2	11·2
38·8	6·7	...	...

the cases, the value of V was less than one-third of that of $\bar{x}$. If the counts approximated to samples of the Poisson series, the values of V should have ranged on either side of the corresponding values of $\bar{x}$. Since they were distinctly lower, it may be inferred that the counts of *Actinomyces* were distributed in the manner of a normal population.

A study of the various operations involved in the plating showed that since (1) the error of sampling—which, in the above case, had been avoided—should have been merely due to random selection from a normal population, (2) the dilutions were carried out carefully and involved only a small experimental error, (3) the colonies of *Actinomyces* appeared on the plates without interfering with each other or with the other organisms and (4) all the colonies were counted, the plating technique, as applied to the counting of *Actinomyces* involved no risk as observed in the cases of random samples of the Poisson series.

The essential difference between the techniques of counting *Actinomyces* and bacteria lies in the fact that, whereas in the former all the colonies appearing on the plates are counted, in the latter only those appearing on certain segments of the petri-dish are counted.

Although under ideal conditions, the bacteria should be distributed evenly over the plates the numbers appearing on individual segments of petri-dishes will be proportional to the areas covered by

them, yet it is not realised in practice and there is always the risk of their not settling in the required numbers over the particular areas that are counted.

In the course of his study of the distribution of yeast cells over the counting area of the hæmocytometer, it was observed by 'Student' (*Biometrika*, 1907, **5**, 351) that the numbers occurring on the individual squares were samples of the Poisson series. If with the same suspension of yeast cells, different sets of the hæmocytometric counts be made, the numbers of cells settling on individual squares in the different sets will be related to each other as samples of the Poisson series. A similar distribution was also observed (Fisher, Thornton and Mackenzie, *loc. cit.*) with regard to the settling of bacteria not only on the individual segments of one petri-dish, but also on those of a number of other dishes when all of them were plated from similar suspensions of bacteria. It being difficult, in practice, to count all yeast cells settling on hæmocytometer, or bacteria on petri-dish, the element of 'risk' arises, obviously, from the need to reckon totals by multiplying by the necessary factors the counts obtained for only a few representative squares or segments as the case may be. Since according to the proposed technique every one of the colonies of *Actinomyces* appearing on the plates was counted, the 'risk' was avoided and the numbers appearing on parallel plates were distributed in the manner of samples of the normal series.

Influence of medium composition on distribution.—Platings were made with the Chirakkal soil on soil extract agar (Fisher, cited from Waksman, *loc. cit.*), the details being the same as in the previous experiment. The values of $\bar{x}$ and V obtained for the different sets of counts (Table XVII) showed that (1) the averages ($\bar{x}$) were not consistent, particularly for suspensions in saline solution, and (2) the variations V exceeded those indicating accuracy sufficient to detect small

TABLE XVII.

Saline Solution		Tap-Water	
$\bar{x}$	V	$\bar{x}$	V
30·3	51·0	36·0	12·0
36·2	16·5	30·0	5·5
37·3	19·0	38·2	85·4
39·0	51·5	37·2	77·5
43·0	150·5	34·8	31·2

changes in numbers. It may be inferred that (1) although the plates may be made with great care, the colonies of *Actinomyces* will not be evenly distributed if an unsuitable medium is used, and (2) the use of the standard medium in conjunction with proper methods of dilution and pouring is essential to obtaining consistent results.

It may be noted that the averages ($\bar{x}$) recorded in Table XVII were highly disproportionate to the corresponding figures in Table II. From the latter it may be gathered that the soil extract medium was so unfavourable to *Actinomyces* present in soils A, B, C and D that no more than one or two poorly formed colonies were observed on the plates. On the other hand, when used for plating the Chirakkal soil (Table XVII), the same medium yielded counts which, though inconsistent, were never below thirty per plate.

To determine whether the observation was due to the previous nutrition and condition of the cells (Subrahmanyan, *loc. cit.*), platings were made of soils A, B, C, D and the one from Chirakkal on mere tap-water set with 2·5 per cent. agar. It was observed that as in the case of soil extract agar practically no colonies appeared on plates made from A, B, C and D, while those from the Chirakkal specimen again averaged over thirty per plate. Since the tap-water could have contained only a small quantity of mineral matter, it may be inferred that the main nutrition of the cells was derived only from reserve materials which they had stored in the soil from which they came.

The foregoing observation and other similar ones referred to in the earlier part of the paper may be regarded as instances of a highly important aspect of microbial nutrition which has not so far received much attention and which, if fully studied, will help considerably in the proper understanding of the physiology of micro-organisms.

SUMMARY.

1. Most of the media commonly used for counting *Actinomyces* by plating were unsatisfactory. A starch medium containing calcium carbonate and minerals was found useful and offered prospects of being improved.

2. The clarity of the medium and the counts of *Actinomyces* were increased, and the undue growths of bacteria and fungi were suppressed, by effecting the following changes in the composition of the original medium:—(*a*) Reducing the quantities of starch, nitrogen and minerals to a minimum, (*b*) replacing calcium carbonate with calcium chloride, potato starch with soluble starch and ammonium sulphate

with a mixture of asparagine and sodium nitrate, and (*c*) maintaining the reaction at P_H 7·4. A medium composed of (in grams), soluble starch, 2·0; HK_2PO_4, 0·5; $MgSO_4$, $7H_2O$, 0·2; $CaCl_2$, 0·05; $Fe_2(SO_4)_3$, traces; $NaNO_3$, 0·05; asparagine, 0·05, in distilled water, 1000 c.c., with washed agar, 20–25 gms. was used. Final P_H 7·4 was developed and found to give satisfactory results.

3. Details relating to preparation of the medium, making of plates, temperature and period of incubation, and counting were standardised.

4. Studies on the consistency of the counts and distribution of colonies on the plates showed that the method developed was highly reliable.

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