FIG. 8.—Frog on left injected with 0·4 c.c. 1 per cent. calcium lactate—10 minutes later 1 mgm. methylguanidin. Frog on right injected 0·4 c.c. Ringer's solution. 10 minutes later, 1 mgm. methylguanidin. (Injections in dorsal sac.) Photographed 50 minutes after injection of the methylguanidin.

FIG. 9.—From frog injected with 0·5 c.c. 1 per cent. $CaCl_2$ 5 minutes after, 0·5 c.c. 0·1 per cent. guanidin (1 mgm.). (× 75.) Killed one hour later.

FIG. 10.—From frog injected with 0·5 c.c. Ringer's solution. 5 minutes after, 0·5 c.c. 0·2 per cent. guanidin (1 mgm.). (× 75.) Killed one hour later.

The Formation of Indigotin from Indol by Soil Bacteria.

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(Communicated by Sir John Russell.—Received October 5, 1927.)

[PLATE 21.]

Indol (C_6 H_4 CH : CH . NH) has a wide distribution in nature, being present in several plants and also produced by putrefactive changes in the intestine. There is no evidence as to its fate after decomposition in soil or in sewage. Its non-persistence at the moderately high temperatures of the manure heap may be due to its volatility ; its disappearance from sewage and manured soil may have to be assigned to some other cause.

Supniewski* states that *Bacillus pyocyaneus* can decompose indol with the production of anthranilic (*o*-aminobenzoic) acid, although the action is extremely slow. In attempting to isolate from soil by the usual selective method of cultivation bacteria capable of destroying indol, it was found that in mineral salt solution containing indol, the latter rapidly disappeared, some colourless compounds being formed whose nature has not been determined. Two organisms have, however, been isolated that can decompose indol with the formation of blue crystals. The chief cultural characters of these two organisms are described below.

1. *Pseudomonas indoloxidans* n. sp. *Morphology* in nutrient broth : straight cells 1 μ to 9 μ long, majority about 3 μ long by 1 μ broad. Motile, with 1 to 4 short curved polar flagella (see fig. 1). The vegetative cells are Gram negative but take the usual dyes. Endospores are not formed. The organism is aerobic and grows best at 25° C. to 28° C.

* 'Biochem. Zeitschr.,' vol. 146, p. 522 (1924).

Nutrient agar colonies, (peptone 0·5, lemco 0·3, agar 1·5 per cent.). *Surface colonies*, (2 days at 28° C.) 1 to 2 mm., round ; convex ; white ; watery ; transparent border, structure radiate, edge erose. (5 *days*) 4 to 5 mm. irregularly round ; slightly convex ; white ; slightly ringed and radiate, shining ; wide translucent border ringed, radiate ; edge undulate. *Deep colonies*, (2 *days*) lens-shaped, brown, slightly irregular. (5 *days*) oval, brown, edge slightly cleft by radiate structure, or lens-shaped.

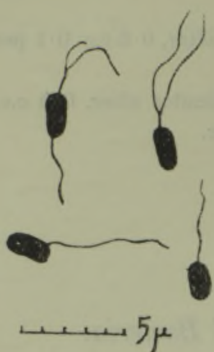


FIG. 1.—*Pseudomonas indoloxidans*, 24 hours old culture on nutrient agar.

Nutrient agar slope (3 *days*) filiform ; convex whitish ; smooth shining ; clear border, undulate.

Nutrient gelatine colonies (peptone 1·0, lemco 0·5, NaCl 0·5, gelatine 12 per cent.). *Surface colonies*, (8 *days* at 14° C. to 19° C.) 1 mm., round ; convex ; light buff ; smooth, shining ; edge erose. Under $\frac{1}{3}$-inch objective, structure appears grumose, deeper part rosettiform. *Deep colonies*, rosettes, erose edge.

Nutrient gelatine stab. Nail head.

Nutrient and *peptone broth*, growth cloudy ; indol is not produced.

Carbohydrate broths, (peptone 1·0 per cent., and 1·0 per cent. of the carbon compounds, dextrose, sucrose, lactose, maltose and glycerol). Growth cloudy but no acid or gas produced ; the media become more alkaline than uninoculated controls.

Starch, (nutrient agar + 0·2 per cent. soluble starch). (16 *days*) no diastase produced.

Nitrates, (nutrient broth + 0·1 per cent. KNO_3). (3 *days*) reduced to nitrites. No gas produced and no growth in the closed arm of the fermentation tubes by the sixteenth day.

Aromatic compounds, phenol and *m*-cresol are not attacked.

Indol, decomposed with formation of blue crystals in mineral salt solution and agar medium.

Indol agar, surface colonies, (3 *days*) 0·9 mm. to 1·0 mm., flat, whitish intermixed with blue, with central opaque nucleus. Blue particles are found up to about 3·75 mm. from edge of colony. (10 *days*) 2 to 3 mm. diameter ; round ; convex ; white, blue tinged ; smooth, shining ; edge erose ; colony mixed with and surrounded by opaque hour-glass shaped particles. *Deep colonies*, (10 *days*) lens-shaped, brown with blue core and surrounded with blue particles. (22 *days*) in thicker gels the edge of the colony is produced to form a flattened whitish ring.

Indol agar slope. First traces of blue are seen within 20 hours, before any growth is visible.

Indol agar stab. After 48 hours the blue zone extends down to about 5 mm. from the surface.

Isolation. One strain from a soil sample obtained from the Italian Tyrol. (Thanks are due to Miss M. D. Glynne, M.Sc., for having collected this sample.)

2. *Mycobacterium globerulum* n. sp. *Morphology* in nutrient agar: curved cells 2 μ to 9 μ long, majority about 5 μ long and 1 μ broad (see fig. 2) breaking up into chains of coccoid cells about 1 μ diameter (see fig. 3). Non motile.

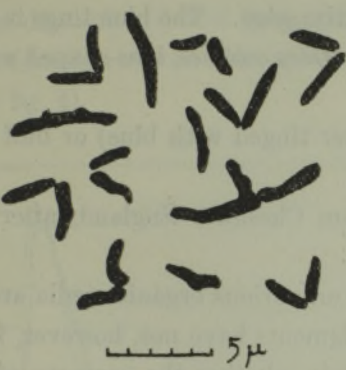


FIG. 2.—*Mycobacterium globerulum*, 20 hours old culture on nutrient agar.

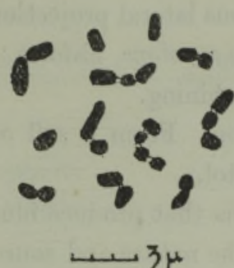


FIG. 3.—*Mycobacterium globerulum*, after 3 days growth on nutrient agar.

Cells are Gram positive but not acid-fast. Capsules can be demonstrated by Muir's capsule method (see Plate 21, fig. 1). On indol agar some cells are surrounded by blue pigment, giving the appearance of capsules (see Plate 21, fig. 2). Endospores are not formed. The organism is aerobic and grows best at 25° C. to 28° C.

Nutrient agar. Surface colonies, (4 *days*) 3 to 5 mm., irregularly round; convex; white; smooth, shining; edge undulate, erose. (7 *days*) more convex and of a watery appearance. *Deep colonies,* (4 *days*) lens-shaped.

Nutrient agar slope, (3 *days*) filiform; flat; watery; edge irregular.

Nutrient gelatine, (19 *days*). *Surface colonies,* 1 to 2 mm., irregularly round; convex; light buff; smooth, shining; edge entire. *Deep colonies,* round, with entire edge.

Nutrient gelatine stab, (8 *days*) nail head, irregularly round; convex; pinkish white; smooth, shining; line of stab erose.

Nutrient and peptone broth, growth cloudy, with viscous suspension. Indol is not produced.

Carbohydrate broths, growth is cloudy in dextrose, sucrose and glycerol, clear with scum or granular suspension in lactose and maltose. No acid or gas produced from any of the compounds.

Starch, (16 *days*) diastase is produced.

Nitrates, (3 *days*) no nitrite or gas produced. No growth occurs in closed arm of fermentation tube.

Aromatic compounds, phenol is attacked, but *m*-cresol is not attacked.

Indol, blue particles are produced on indol agar but not in solution.

Indol agar, *surface colonies*, (4 *to* 7 *days*) 1 to 2 mm., round ; convex ; white, tinged with blue ; smooth, shining ; entire edge. The blue tinge is chiefly due to discrete hour-glass shaped particles. *Deep colonies*, lens-shaped with bunchy filamentous lateral projections.

Indol agar slope, filiform ; white (later tinged with blue) or buff to yellow ; smooth, shining.

Isolation. From a soil obtained from Cheshire, England, after treatment with scatol.

Bacteria that produce blue pigments on various organic media are known to occur ; the nature and source of the pigments have not, however, in any case been made known. In order to ascertain whether the property of producing a blue pigment on synthetic media containing indol was possessed by other known bacteria, slope cultures of 74 strains of soil bacteria were made on a medium consisting of mineral salt agar with glycerol 0·1 per cent., $(NH_4)_2SO_4$ 0·05 per cent., and indol 0·005 per cent. Very few of these made any appreciable growth in 7 days at 27° C. Only *Ps. indoloxidans* and a new species of *Micrococcus** formed blue particles, but this latter species has not so far formed them in solution cultures. It was decided, therefore, further to investigate the nature of the blue substance produced in cultures of *Ps. indoloxidans*.

The Nature of the Pigment.

The blue compound that appeared in cultures containing *Ps. indoloxidans* was found as insoluble particles ; their identity with indigotin was suggested by the following tests† :—

1. Insolubility in water, alcohol, ether, xylol, benzol.
2. Dissolved in strong sulphuric acid to give a blue solution.
3. This solution, on dilution, dyed silk blue.

* A description of this organism (*Micrococcus piltonensis*) is in course of publication in 'Centralblatt für Bakteriologie,' II. Abt., Bd. 73.

† I am indebted to Dr. F. Tattersfield for assistance in these tests ; the spectrophotometry was carried out by Dr. S. Judd Lewis, Staple Inn Buildings, London.

4. The colour disappeared on treatment with reducing agents and caustic soda, but returned on pouring into water and on exposure to air.
5. The colour was discharged by nitric acid.
6. A solution in concentrated H_2SO_4 was treated with strong soda, whereupon a green colour appeared; the colour went to yellow on addition of more soda; this yellow then went to green on the addition of ammonia.
7. Spectrophotometric analysis of a solution prepared from some of the dry material with concentrated H_2SO_4 produced a curve for the extinction coefficient that was so similar to that obtained from a solution of indigotin di-sulphonic acid prepared from pure powdered indigotin that the identification of the blue substance as indigotin is placed beyond doubt (see fig. 4).

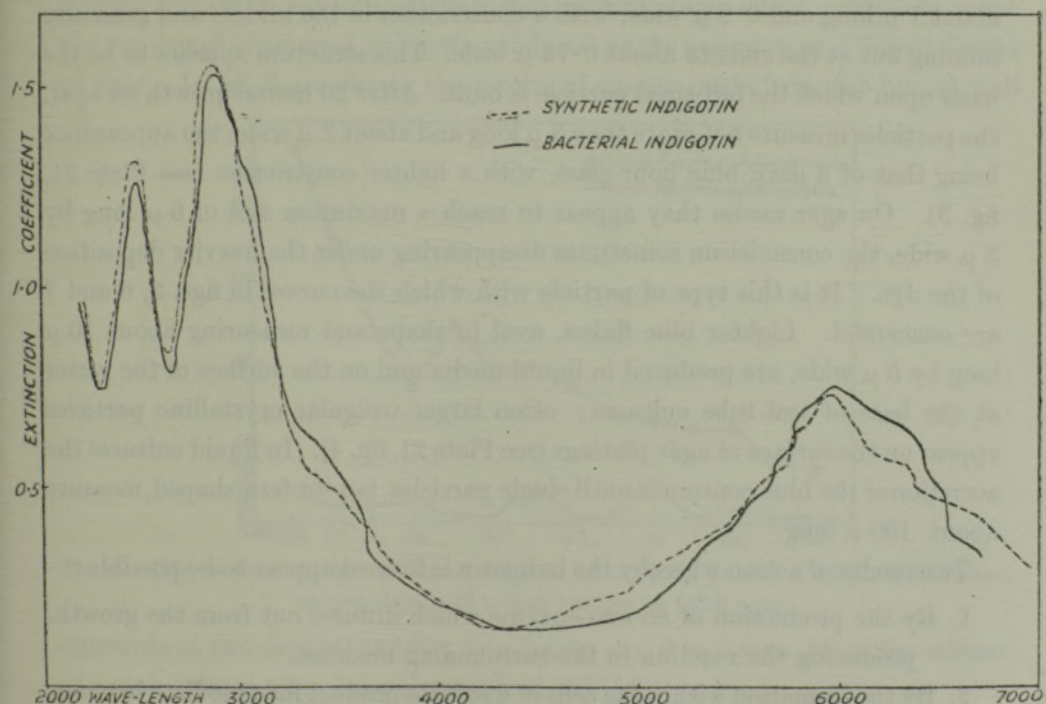


Fig. 4.—Comparative curves for extinction coefficient of synthetic indigotin and indigotin produced from indol by *Ps. indoloxidans.*

The Mode of Formation of Indigotin on an Agar Medium.

Pure indol, melting point 52° C., obtained from the British Drug Houses, was added to a mineral salt agar, after the latter had melted, until the indol was completely dissolved. The following were the salts used in the medium :—

	Per cent.		Per cent.
K_2HPO_4	0·100	$CaCl_2$	0·010
$MgSO_4 . 7H_2O$......	0·020	$FeCl_3$	0·002
NaCl	0·010		

To this medium carbon and nitrogen sources were added as glycerol 0·1 per cent. and $(NH_4)_2SO_4$ 0·05 per cent. respectively. The concentration of indol was 0·01 per cent. The medium was then filtered through cotton-wool, tubed, and sterilised in the autoclave at 15 lbs. pressure for 15 minutes.

The general appearance of the growth on indol agar has already been described (p. 264). The particles are first visible as thin needle-shaped blue crystals, about 1 μ long and 0·2 μ wide, with a constriction in the middle and generally fanning out at the ends to about 0·75 μ wide. This structure appears to be the basis upon which the full-sized particle is built. After 20 hours' growth on agar, the particles measure not more than 3 μ long and about 2 μ wide, the appearance being that of a dark blue hour-glass, with a lighter constriction (see Plate 21, fig. 3). On agar media they appear to reach a maximum size of 6 μ long by 3 μ wide, the constriction sometimes disappearing under the heavier deposition of the dye. It is this type of particle with which the curves in figs. 5, 6 and 7 are concerned. Lighter blue flakes, oval in shape and measuring about 10 μ long by 5 μ wide, are produced in liquid media and on the surface of the water at the base of test-tube cultures; often larger irregular crystalline particles appear on the surface of agar platings (see Plate 21, fig. 4). In liquid cultures the accretion of the blue continues until single particles, fan- or fern-shaped, measure about 150 μ long.

Two modes of action whereby the indigotin is formed appear to be possible :—

1. By the production of an exo-enzyme which diffuses out from the growth, producing the reaction in the surrounding medium.
2. By the formation within the cells of a soluble product intermediate between indol and indigotin which diffuses outward and is then converted into indigotin.

Indoxyl, a product intermediate between indol and indigotin, is an unstable compound and is very rapidly oxidised to indigotin on exposure to atmospheric oxygen. On the second hypothesis, if indol were taken in by the organism and excreted as indoxyl, it would be expected that each bacterial cell would become surrounded by a blue " capsule " of indigotin, in the way that has been described

(p. 266) as happening sometimes to cells on indol agar slope cultures of *Mycobacterium globerulum*. This does not take place with *Ps. indoloxidans*, nor does indigotin occur inside the cells under what appear to be the optimum conditions for oxidation. It seems unlikely, therefore, that indoxyl is formed within the cells of this organism.

Production of Indigotin in Agar Stab Cultures.

The production of indigotin in stab cultures is confined chiefly to the top 3 to 5 mm., and extends radially from the line of stab to the walls of the tube. No increase in intensity of blue or depth to which the blue extended has been observed to take place after about the third day. Although the organism continues to grow to some extent on the surface of the stab, it is found that a considerable growth of the organism also occurs along the line of stab. By cutting transverse sections across a stab culture, it was found possible to count the number of crystals of indigotin produced by the organism at different depths of agar. In fig. 5 are shown the results of counts made from the line of stab

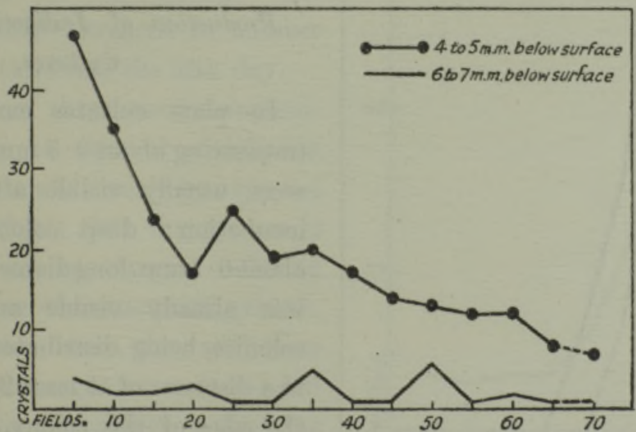


Fig. 5.—Curves showing the number of particles of indigotin produced by *Ps. indoloxidans* at two depths of a stab culture on indol agar.

outwards at two depths, namely, 4 to 5 mm. and 6 to 7 mm., from the surface of a stab culture after 2 days' growth at 25° C.*

* The particles were counted as follows. The agar was removed from the test-tube by breaking the latter and a section about 2 mm. thick cut at the fourth to fifth millimetre from the surface. This section was applied to a coverslip and the latter inverted on the mechanical stage of the microscope. The $\frac{1}{6}$-inch objective was focussed on to the edge of the bacterial growth; a cover slip upon which had been marked a rectangle measuring 3 mm. square was placed in the ocular. The number of particles of indigotin within the rectangular area at 50 μ from the surface of the cut down to a depth of 100 μ was counted; the stage was then moved so that the adjoining field was brought into register and the particles counted as before; these operations were repeated along one traverse

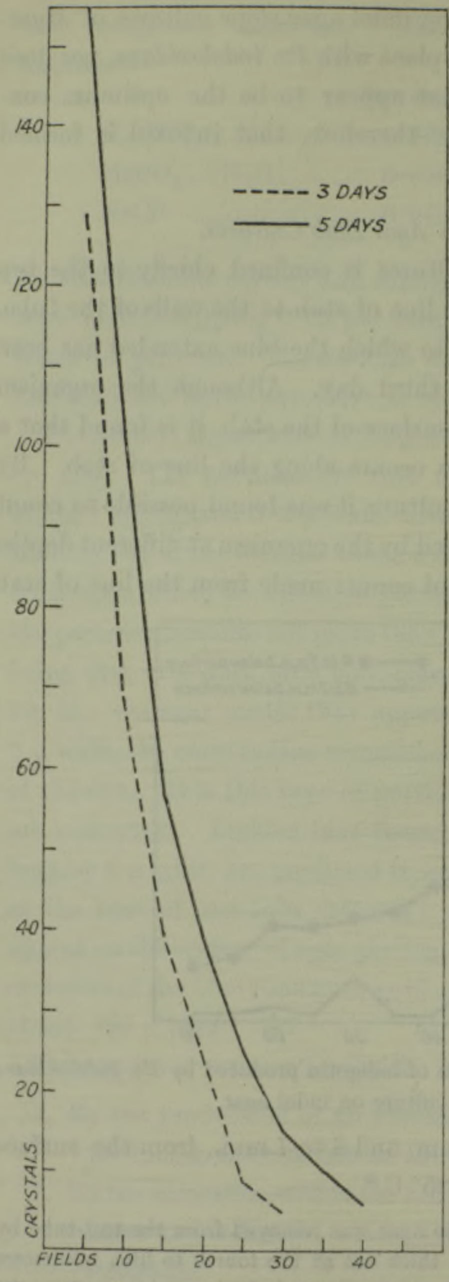


FIG. 6.—Curves showing the number of particles of indigotin produced by a colony of *Ps. indoloxidans* on the surface of indol agar in a petri dish after 3 days' and 5 days' growth.

The upper curve confirms the observation made, that more indigotin is produced near the bacterial growth. The lower curve suggests that crystals produced at some distance from the line of stab are due to the activity of the organisms on the surface. Apparently the organisms cannot initiate the process at the lower depth owing to insufficiency of oxygen, but once they have initiated it at the surface they can form crystals at this depth. This supports the hypothesis that the action is due to the production of an exo-enzyme diffusing out from the bacterial growth.

Production of Indigotin in Plate Cultures.

In plate cultures surface colonies (measuring about 0·5 mm. in diameter) were usually visible after 48 hours' incubation; deep colonies measured about 0·1 mm. long diameter. Indigotin was already visible around surface colonies, being distributed horizontally to a distance of at least 2·25 mm. from the edge of the colony. The largest particles measured 3 μ to 3·5 μ long, but the majority were too small to enable a count to be made with sufficient accuracy. The growth of the organism was stopped by exposing the plate to the vapour of formaldehyde; examination made at intervals for some days showed that production of indigotin had also ceased. The colonies on a

up to the edge of the agar. The section was then reversed and the particles counted at 50 μ to 100 μ below the surface of the lower cut. In drawing up the curves the number of particles of indigotin in every 5 fields was added together in order to smooth out differences due to inequalities of the gel.

plate culture not so treated continued to produce indigotin to the maximum distance. A count was made on a series of plates on which the production of indigotin took place between the forty-eighth and sixty-sixth hours. The colony chosen was 0·95 mm. in diameter, and three traverses along horizontal columns of agar each 50 μ thick were made in one direction to a distance of 3·3 mm. from the edge of the colony. The result of this count is shown by the broken line in fig. 6. Counts were made from this colony two days later (the colony having then grown to 1·2 mm. in diameter), in order to ascertain if any increased oxidation had taken place. The result is shown by the unbroken line, the points on which represent the mean of two traverses on opposite sides of the colony. The particles slightly increased in number between the third and the fifth day.

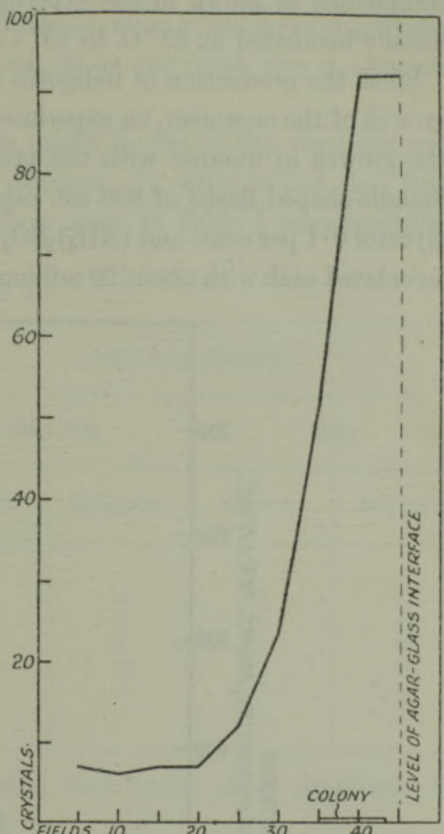


Fig. 7.—Curve showing the number of particles of indigotin produced by a colony of *Ps. indoloxidans* situated near the floor of the petri dish culture. Semi-diagrammatic.

The shape of these curves approximates closely to that of a diffusion curve. This is what would be expected on the hypothesis that the process is effected by an exo-enzyme diffusing outwards from the bacterial growth. If, on the other hand, the indol were taken in by the organism and excreted as indoxyl the probability is that the oxidation of the latter, as soon as it reached an area of higher oxygen-tension outside the colony, would appear as a thick zone of blue particles close to the colony. This, however, does not take place at any stage of growth. Furthermore, colonies buried at the bottom of a 5-mm. thick layer of indol agar produced a similar curve for the vertical distribution of indigotin, the maximum amount being formed at the shortest radius from the colony (see fig. 7).

The Carbon and Nitrogen Requirements of Ps. indoloxidans.

In these experiments the same mineral salt solution was used as in agar cultures. Except when amino-compounds were supplied, ammonium sulphate was generally given as the source of nitrogen, since the production of free nitric acid from nitrates might cause a loss of indigotin. Carbon was supplied by the compounds as shown in the experiments reported below. The cultures were usually incubated at 22° C. to 25° C.

Since the production of indigotin appeared to be associated with the active growth of the organism, an experiment was carried out to correlate, if possible, the growth in number with the production of indigotin in solution cultures. Spindle-shaped flasks of 300 c.c. capacity containing 100 c.c. of solution with glycerol 0·1 per cent. and $(NH_4)_2SO_4$ 0·05 per cent. and 10 mgms. of indol were inoculated each with about 20 millions of cells per flask and incubated for 9 days

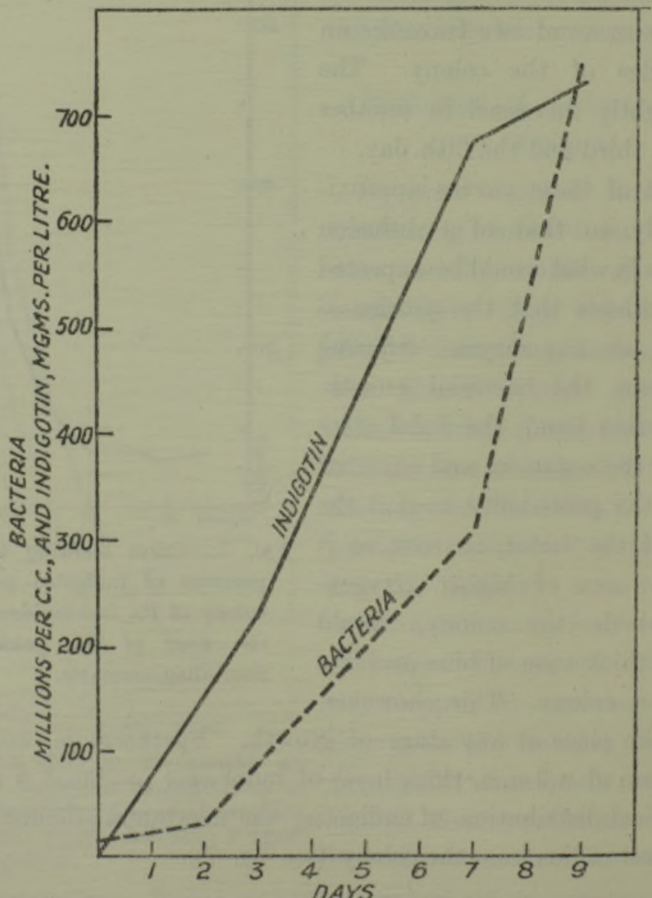


Fig. 8.—Curves showing bacterial numbers and amount of indigotin produced.

at 25° C. A blue tinge due to the indigotin was visible on the second day, when a hæmocytometer count of the total bacterial cells showed 30 millions per cubic centimetre.

The result of the analyses for indigotin* and the bacterial counts are shown in fig. 8. Indol was present, as shown by the pink colour given with Ehrlich's dimethyl-*para*-amino-benzaldehyde reagent, on the third and seventh day, but had disappeared on the ninth day. The production of indigotin is associated with rising bacterial numbers; the falling off on the ninth day is associated with the disappearance of the indol.

It seemed possible that this depression might be due to the organism using indol as a source either of energy or of nitrogen. In order to test this, flasks of basal media containing the compounds as given in Table I were inoculated and incubated for 3 days.

Table I.

	Additional nitrogen.			
	$(NH_4)_2SO_4$.		Nil.	
	Growth.	Indigotin.	Growth.	Indigotin.
Carbon compound—				
1. Indol	0	0	0	0
2. Glycerol	+	0	0	0
3. Tryptophan	+	0	+	0
4. Glycerol + tryptophan	+	0	+	0
5. Glycerol + indol	+	+	+	+
6. Tryptophan + indol	+	+	+	+

From this experiment it was concluded that under these conditions the organism—

(*a*) cannot use indol as a source of energy;

(*b*) cannot oxidise indol to indigotin without an additional source of carbon;

(*c*) can oxidise indol (given a supply of carbon) without an additional supply of combined nitrogen, and thus probably obtains nitrogen from the indol.

An analysis of the medium, without indol or ammonium sulphate, showed that the amount of nitrogen present was only 0·0000025 per cent., which is considerably less than the analytical error.†

* The method used for estimating the amount of indigotin was essentially Rawson's, as described by W. A. Davis in 'Agric. Res. Inst. Pusa' (Indigo Publications), 1921.

† The error was about 0·000025 per cent. I am indebted to Dr. H. L. Richardson for this estimation by the Kjeldahl method.

It is interesting to note from Table I that the organism cannot convert tryptophan into indigotin; neither indol nor scatol was at any time produced. Scatol is not attacked by the organism.

A high C : N ratio appears to be most suitable to the growth of *Ps. indoloxidans* and the production of indigotin. The following amounts of glycerol and ammonium sulphate were added to 100 c.c. of the mineral salt solution:—

	Glycerol.	$(NH_4)_2SO_4$	Ratio. C	Ratio. N
	gm.	gm.		
Culture *a*	0·5	0·0126	16	1
,, *b*	0·125	0·0126	4	1
,, *c*	0·031	0·0126	1	1

The above quantities were added from sterile solutions to the autoclaved mineral salt solution containing the indol. After 8 days' incubation at 25° C., indol still

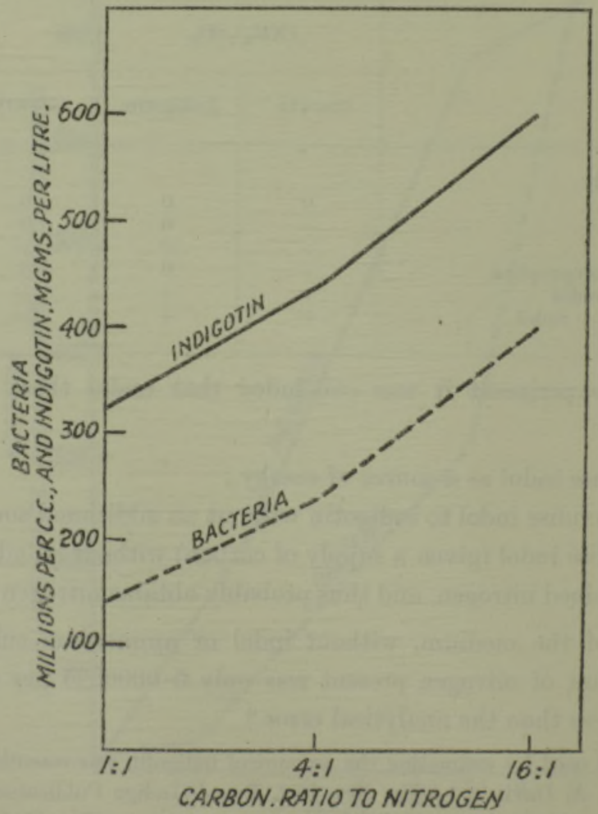


Fig. 9.—Curves showing the relation between bacterial numbers and amount of indigotin produced at different ratios of carbon to nitrogen.

occurred in flask (*c*), but not in the other two. The total bacteria and the amount of indigotin produced are shown in fig. 9. The bacterial numbers and the amount of indigotin produced both increase with higher ratios of carbon to nitrogen.

Availability of Organic Compounds.

In order to discover the relative value of different carbon compounds as sources of energy, flasks of media containing ammonium sulphate as the additional source of nitrogen, were set up with the carbon compounds shown in Table II in the concentration 0·1 per cent. (except the media containing no additional carbon compound, that with cellulose, and that with naphthalene). The concentration of indol was 0·01 per cent. The cloudiness or otherwise of the solution and the time when the blue of the indigotin was first noted are indicated.

Table II.—Relative Availability of the Different Sources of Energy.

A—The following compounds did not supply energy to the organism and indigotin was not produced:—

Methyl alcohol, amyl, capryl, formic, sodium formate, calcium formate, ammonium oxalate, valerianic acid (normal), xylose, sucrose, cellulose, phenol, naphthalene, indol.

B—Compounds supplying energy to the organism.

Indigotin produced.			
Solution clear. (Growth slight.)	Blue first noted.	Solution cloudy.	Blue first noted.
	Days		Days.
		Ethyl alcohol	2
		Propyl alcohol, normal	5
		Propyl alcohol, iso	5
		Butyl alcohol, normal	3
		Butyl alcohol, iso	3
		Acetic acid	3
		Propionic acid	3
		Butyric acid	3
		Butyric acid, iso	3
		Valerianic acid, iso	3
Adonitol	12	Glycerol	2
Dulcitol	14	Mannitol	5
Arabinose	13	Dextrose	3
Galactose	5	Lævulose	5
Lactose	13		
Maltose	13		
Raffinose	19		
Starch	20	Glycollic acid	3

Glycerol was thus the most suitable of the compounds tested for supplying the organism with an easily assimilable source of energy. It is of interest to note that the organism behaves differently towards closely related compounds, *e.g.*, xylose and arabinose, and the *normal* and *iso* forms of valerianic acid.

The relative value of certain amino-compounds as energy sources was also tested. In this case, the ammonium sulphate was omitted from the solutions and no additional source of energy was supplied.

Table III.—Relative Value of certain Amino-Compounds as Sources of Energy to *Ps. indoloxidans*.

No indigotin produced.	Indigotin produced from indol with	Blue first noted.
		Days.
Acetamide	Urea	8
	Asparagine	4
	Glycine	4
Cystine	Alanine	4
Valine	Tyrosine	8
	Tryptophan	1
o-Anthranilic acid	Aspartic acid	1
Isatin		

Growth was good on all of the media in which the indigotin was produced, but of the other compounds cystine alone supported any growth.

Purity of the Indigotin.

The indigotin as collected on the asbestos filter bed previous to sulphonation is mixed with a considerable amount of impurity, *e.g.*, mineral salts precipitated in the medium and bacterial growth. The amount of crude indigotin collected from cultures in which the bacteria had completed or passed their period of maximum growth is a fairly constant one, about 20 to 35 milligrams per 100 c.c. of culture. The presence of these impurities may affect the curve obtained by spectro-analysis, especially in the visible region. The following are a few of the results obtained from the analysis of about 40 cultures.

The figures in the final column represent the amount of indigotin produced expressed as a percentage of the amount of indol supplied. Since, however, 112 gms. of indigotin may be produced from a 100 gms. of indol, the percentage figures must be divided by 1·12 to arrive at the percentage of efficiency of the oxidation.

The cultures quoted in Table IV, with the exception of No. 5, were grown at 23° C. to 25° C., but were not parallel cultures. No. 5 consisted of 500 c.c. medium in a Woulff's bottle, and was grown with a current of air flowing through continuously for the time stated at 10° C. to 12° C. (room temperature).

Table IV.—Indigotin produced by Cultures of *Ps. indoloxidans* in Solutions with various Sources of Energy and Nitrogen.

Carbon.	Nitrogen.	Age.	Crude indigotin.	Pure indigotin per cent. of crude.	Indigotin per cent. of indol supplied.
			mgms.		
1. Glycerol	$(NH_4)_2SO_4$	3	9·0	28·6	25·7
2. ,,	,, ...	16	10·5	60·7	63·7
3. ,,	,, ...	19	21·0	39·7	63·3
4. ,,	α-amino propionic acid	18	24·9	30·2	75·3
5. ,,	asparagine, KNO_3	10	140·5	33·5	94·1
6. Amino-acetic acid		9	24·0	27·6	66·2

Efficiency of Oxidation.

In the experiments quoted above the best conditions for a maximum yield of indigotin may not have been found. While the indol can supply nitrogen and thus reduce the amount of indigotin produced, some of the indol may have been lost by volatilisation during sterilisation. An experiment was therefore set up, having the indol melted in the glycerol ammonium sulphate medium after the latter had been sterilised. The inoculated flasks were incubated at 25° C. for 4 days, when the bacteria in 1 c.c. from each flask were counted by plating on nutrient agar, and the remainder of the culture filtered to collect the indigotin. The result was as follows :—

	Bacteria, millions per c.c.	Indigotin, mgms.
Culture 1	268	11·39
,, 2	298	9·17

The first of these two cultures gave the highest percentage conversion (100 per cent.) of the indol that has been obtained. The efficiency of oxidation can, therefore, be raised by this procedure.

Toxicity of Indol.

Indol appears to depress the growth of the organism. This was tested by growing the organism on media containing the usual amount (0·01 per cent.) of indol, and half of that concentration. A comparison was made at the same time between glycerol and dextrose as sources of energy. The bacteria were counted by plating on nutrient agar after 4 days' incubation at 25° C. The result is shown below.

Growth of *Ps. indoloxidans* at Two Concentrations of Indol.

Indol. per cent.	Bacteria, millions per c.c. Dextrose.	Glycerol.
0·005	327	237
0·01	22·5	25

The higher concentration of the indol therefore prevents rapid growth of the organism. It was also noted from this and other experiments carried out under similar conditions that the organisms multiplied rapidly as soon as the indol had been used up.

Delayed Addition of Indol.

The effect of adding indol to a culture after growth had started was also tested. Flasks of mineral salt solution of the usual composition, with glycerol, were inoculated and incubated at 27° C. After 2, 4 and 7 days' growth 10 c.c. of a 0·1 per cent. solution of indol was run into the cultures. Blue crystals appeared within 24 hours in the cultures thus treated after 2 days, but not in those treated after 4 and 7 days' growth. The production of indigotin, however, after 2 days' growth, was not continued to any extent. It appears, therefore, that the oxidation of indol takes place only during the early stages of growth of the organism.

Intermediate Products.

If the conversion of indol to indigotin by *Ps. indoloxidans* is a simple oxidation, indoxyl would be expected to occur as an intermediate product. It has, however, been suggested (see pp. 268–269) that it is improbable that the excretion of indoxyl takes place from within the cells of *Ps. indoloxidans*. Davis* states that the presence of unoxidised indoxyl in the liquor of the beating vat may be detected by exposing a little of the solution on filter paper to the fumes of ammonia; if free indoxyl is present the paper becomes blue or green. No trace of such a reaction has been found in several cultures of *Ps. indoloxidans*, all of which remained alkaline; traces of indoxyl would not remain in solution long enough to allow detection by that method. From the further fact that the whole of the indol supplied can be oxidised to indigotin, it also seems unlikely that the presence of free indoxyl could be demonstrated.

Further Oxidation Products.

Since indigotin may be further oxidised to compounds which are soluble in water, and therefore not collected in the precipitate, an attempt was made to

* 'Agric. Res. Inst. Pusa' (Indigo Publications), 1921.

discover the presence of isatin in older cultures. The method adopted to test for the presence of isatin was that given by Porcher.* This consists in acidifying the solution to be tested and shaking it up with ether ; the ether extract is evaporated on a porcelain dish and the residue treated with a few drops of benzol containing 1 per cent. thiophen, when the presence of isatin is indicated by a bright blue rim. No trace of this reaction has been observed in several cultures on which this test was made. It has already been stated that loss of indol by volatility may account for the yield of indigotin being less than that which it is theoretically possible to obtain, and that this loss can be overcome by omitting sterilisation. It seems improbable, therefore, that indigotin is further oxidised to isatin by this organism.

Summary and Abstract.

1. A soil organism, *Pseudomonas indoloxidans*, n.sp., oxidises indol to indigotin in solution cultures and on agar media.

2. The indigotin is formed as crystals outside the organism, appearing first within 20 hours on agar media. The crystals decrease in number with increasing distance from the bacterial growth.

3. Indol does not act as a source of energy, some other source being necessary to effect the oxidation of indol to indigotin. Of a number of carbon compounds tested, glycerol appears most readily to act as a source of energy in the oxidation. Bacterial numbers and the amount of indigotin produced increase with higher ratios of carbon to nitrogen. The organism can utilise trytophan as an energy source but produces neither indol nor indigotin therefrom.

4. Indol is oxidised only by young growing cultures, and can be oxidised in the absence of other nitrogen compounds ; it depresses the multiplication of the bacteria.

5. No trace of indoxyl has been found in cultures, and the indigotin is not further oxidised to isatin.

6. Two other soil organisms, *Mycobacterium globerulum* n.sp. and *Micrococcus piltonensis* (Gray and Thornton) n.sp., can also produce small amounts of indigotin on indol agar only.

I wish to thank Mr. H. G. Thornton, Head of the Department of Bacteriology, for his unfailing interest and valuable suggestions.

* ‘Bull. Soc. Chim. de France’ (IV), vol. 5, p. 526 (1909).

DESCRIPTION OF PLATE 21.

Fig. 1.—*Mycobacterium globerulum*, showing capsules stained by Muir's method. From a camera lucida sketch. (× 1100.)

Fig. 2.—*Mycobacterium globerulum*, showing some of the cells, stained with erythrosin, surrounded by indigotin. From a camera lucida sketch. (× 1100.)

Fig. 3.—Two crystalline particles of indigotin produced by *Pseudomonas indoloxidans*.

Fig. 4.—Photomicrograph of the edge of a surface colony of *Pseudomonas indoloxidans* on indol agar, showing particles of indigotin on the surface of the agar outside the colony.

Fig. 5.—The same, at a deeper focus, showing the hour-glass shaped particles below the surface.

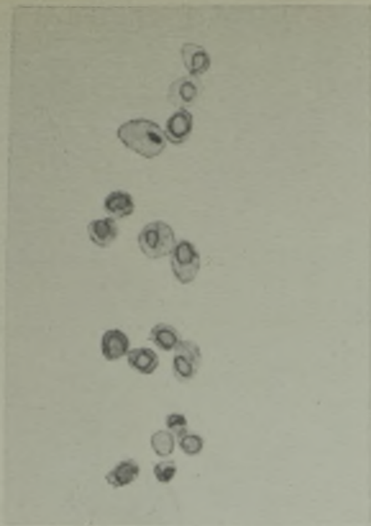
A Consideration of Some Attempts to Analyse Growth Curves.

By G. E. Briggs, St. John's College, Cambridge.

(Communicated by Dr. F. F. Blackman, F.R.S.—Received November 9, 1927.)

No matter what attribute of an organism is considered—height, weight, activity, etc.—the rate of change of this attribute, the growth, depends upon the age of the organism. In many cases the rate of change is small at first, becomes faster and faster for a certain time, and then slower and slower. This drift of the rate of growth with increasing age is found when organisms are kept in constant environments. Such a drift in a constant environment may be taken as an expression of a drift in the organism itself, and the rate of growth can be taken as a measure of this "internal factor." If two precisely similar organisms were placed at their beginnings in two different constant environments, it is highly probable that the form of the drift of growth rate with time would be different. One of the organisms would be older than the other when a certain growth rate was attained.

Although we might say that at these points the intensity of the "internal factor," or the effective amount of "growing material" as it has been called (6), of the two organisms was the same, we do not mean that the organisms are identical. The "internal factor" determining the rate of growth is a kind of integration of all the various factors inside the organism. For example, in the case of the growth in weight of a plant, number and size of growing points, accessibility of food supplies, etc., are factors determining the dimensions of the "internal factor." The two organisms may differ in each of their subsidiary factors whilst possessing "internal factors" of like dimensions.



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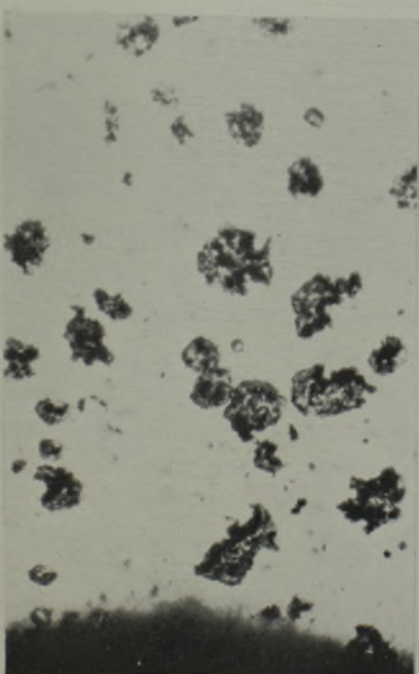

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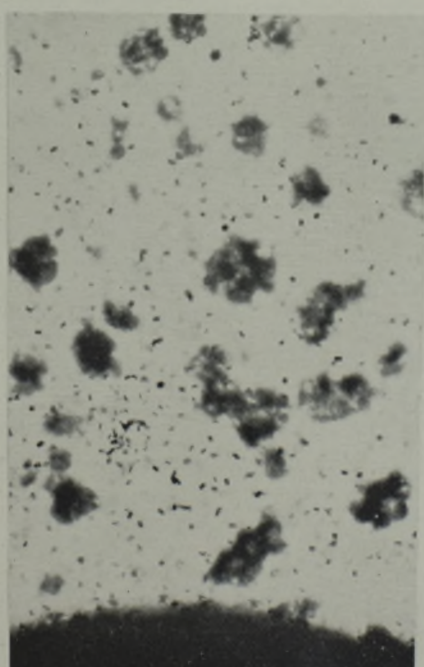

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