UNIVERSITY OF EDINBURGH

A STUDY OF PSEUDOMONAS WITH SPECIAL REFERENCE TO SPECIES PATHOGENIC TO STONE-FRUIT TREES.

by

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Thesis for the degree of Doctor of Philosophy
in the Faculty of Science.
May, 1956.

CONTENTS

GENERAL INTRODUCTION

GENERAL INTRODUCTION

'Diseases are too often taken for granted until irreparable harm has been done. Recognition of their importance is the first step in doing something about them.'

- U.S. Yearbook of Agriculture, (1953).

The present study concerns one of the groups of bacteria which include plant pathogens among their members. The genus Pseudomonas is widely represented in nature, the majority of these organisms being common saprophytes. Only one, Ps. aeruginosa, may be classified as an animal pathogen but a large number of the species are the causal organisms of plant diseases.

Stone fruit trees are subject to attack by certain species of Pseudomonas. The result of such attack is often severe or even fatal to the host. Bacterial canker, die-back, shot-hole and gummosis are among the descriptive terms given to the various manifestations of the disease. Bacterial canker is an appropriate name because the infected areas on branches form necrotic lesions or cankers, causing a common and most destructive phase of the disease. Die-back is the term often associated with the effect of more localised infections occurring on twigs and small branches. Shot-hole describes the nature of the infection when it occurs on leaves; the centres of the small necrotic lesions often drop out producing typical brown-edged perforations.

The/

The term gummosis is suggested by the oozing of gum from parts of infected branches, but this is by no means a certain symptom of the bacterial disease as it is a common reaction of Prunus spp. to various kinds of damage. A less frequent result of infection, at least in Great Britain, is a so-called blossom wilt when the flowers die soon after opening. Occasionally the fruit may be infected producing soft necrotic lesions on the surface tissues.

In Scotland the main stone-fruit crop is the plum. The orchards are located mainly along the valley of the river Clyde focussing on the towns of Carluke and Lanark but are also found scattered over other parts of the Lowlands. There is little statistical information available regarding the values of the crops lost due to this disease but, according to figures obtained (private correspondence) from the Department of Agriculture for Scotland, about 5% of the crop was lost during 1945, mostly as a result of bacterial canker. From personal observation of orchards in the years 1953-55 it would appear that such an estimate would now be unduly low, the figure being nearer to 15%. The following case stresses the disastrous nature of the disease. A grower planted 360 trees (variety "Victoria") in 1952. It is responsibly estimated that if the trees continue to die off at a constant rate the entire batch will have been lost by the eighth year after planting; the time at which profitable yields of fruit are normally expected./

expected. Other growers have had similar experiences and are now regarding the replacement of losses as uneconomic.

Extensive studies have been carried out in England on the nature and control of the disease with particular reference to the cherry although other stone-fruits including the plum have also been given attention. The relevant literature is quoted by Crosse (1953) and Crosse & Bennett (1955). In Scotland, however, little or no work has been done on plum canker. As climatic conditions differ in the two countries it was considered desirable to study certain aspects of the disease with particular reference to local conditions.

Observations of bacterial canker on plum trees.

The well-known variety "Victoria" constitutes the bulk of the plum crop in Scotland. A large percentage of the trees were planted a generation ago but since the last war considerable areas have been restocked. The older and well-established trees generally suffer only minor damage from bacterial canker, infection usually being restricted to occasional branches and twigs. Cankers girdling the main stems of such mature trees are comparatively rare. The mortality rate is much higher with younger trees, i.e. less than eight years old, where, in addition to the twig and branch infections, a greater proportion of cankers develop on and encircle the main stem - a condition which is/

is inevitably fatal.

The seasonal cycle of the disease in England has been defined (Crosse, 1954) as occurring in two main phases, one confined to winter and the other to spring. In the winter phase an infection of the bark produces a progressive deterioration of the tissues which is arrested as the tree regains resistance in the spring. At this stage the organisms in the bark cankers die out. If the canker has girdled the stem the tissue above is deprived of nutrients and is killed. If the stem is not completely encircled by the end of the winter phase the canker usually remains localised and may or may not restrict further development of the tree according to the size and position of the infected area. Before the bacteria disappear from the cankers the young leaves become infected. A further infection of the leaves of the later-formed extension shoots provides the inoculum for the autumn infection of the stems and branches through natural apertures or wounds and cracks in the bark. The leaf scar is said to be a major site of such infection in the cherry but some doubt is expressed about its importance in the case of the plum.

In Scotland it is a common practice to stake and tie the young trees for a number of years after planting. This is often necessary owing to the strong winds prevalent in orchards. It seems that an abrasion of the bark with the tie permits infection at a point where the maximum amount

of/

of damage can ensue. Numerous cases have been examined in which the trees were killed by cankers which appeared to originate from such abrasions.

The causative organism and its identification.

According to Crosse (1954), two closely related but distinct species are involved in Britain. The first, Pseudomonas mors-prunorum Wormald, is responsible for most of the bacterial diseases of cherry, plum, and other species of Prunus. The second species, Pseudomonas prunicola Wormald, occurs occasionally on the cherry and the plum. On the latter its activities are limited to the young, green extension shoots.

Wormald (1932), in his original description of Ps. mors-prunorum, compared it with Ps. prunicola and found a strong affinity between the organisms. They differed, however, in certain particulars, e.g.

(a) in 5% sucrose nutrient broth Ps. mors-prunorum produced a greater degree of opacity.

(b) in 5% sucrose nutrient broth the growth of Ps. mors-prunorum was usually dead in four to six days whereas that of Ps. prunicola remained viable for several weeks.

(c) on nutrient agar with 2% lactose and brom cresol purple Ps. mors-prunorum produced an alkaline followed by an acid reaction whereas with Ps. prunicola the reaction remained alkaline.

Differences/

Differences in the relative amounts of acid produced on lactose and maltose were suggested by Crosse (1953) as additional criteria for distinguishing the two species.

Wilson (1931, 1936) suggested that Ps. prunicola with other organisms causing bacterial canker of stone fruit and blossom-blast of pear should be considered similar if not identical with Ps. syringae (van Hall).

Erikson (1945) in a study of Ps. mors-prunorum and related phytopathogenic bacteria, including Ps. prunicola, concluded that there was no justification in raising them to specific rank. It is of interest here to note that the descriptions of the disease caused by Ps. mors-prunorum in Britain and that caused by Ps. syringae in America and New Zealand also correspond closely.

Taxonomic problems.

The very close relationships existing between the organisms described above illustrate the difficulty encountered in the identification of species of Pseudomonas.

In the writer's own experience the almost synonymous nature of the published descriptions of the organisms make identification an uncertain process. Burkholder & Starr (1948) found it impossible to identify phytopathogenic species by normal bacteriological procedures. They suggest that there may be only a few true species but a great number of/

of formae speciales within the group and point out that the weakness in the classification may be due to the failure of the investigator to recognise which bacteriological characters are generic and which are specific. Gaby (1955) also considered that the genus contained many ill-defined and illegitimate species.

The confusion which thus exists in the classification of the species makes it desirable that at least some of the characters used should be reconsidered. The following laboratory investigations were carried out with the aim of relieving some of that confusion, particularly among the species pathogenic to Prunus spp.

Strains studied.

The cultural investigations which follow were carried out using selections of 204 strains of Pseudomonas from a variety of sources, which allowed a comparison of different plant pathogens, plant saprophytes, and other organisms. Particular emphasis has been laid on a small representative section of the strains (Series I). This section was used mainly for preliminary studies prior to the investigation of the whole series of isolates. The grouping used here is one of convenience and has no cultural or other significance.

SERIES I./

SERIES I. Selected strains.

	Ref. No.	Specific name	Source
A	P 1	syringae	Dowson (Cambridge)
	2	coronafaciens	" "
	3	marginalis	" "
	4	phaseolicola	" "
	5	viridiflava	" "
	6	mors-prunorum	" "
B	8	---	grass
	9	---	grass silage
	10	---	" "
C	11	aeruginosa	stock culture *
	12	---	soil

* This strain is now apyocyanogenic after being in laboratory culture for many years.

SERIES II. Plant pathogens.

The sources of the organisms detailed in this series are considered to be reliable and, as such, are suitable for comparative purposes in this work.

Specific name	Source	No. of strains
mors-prunorum	Crosse (E. Malling)	5
"	Dowson (Cambridge)	1
prunicola *	Crosse (E. Malling)	1
syringae	Dowson (Cambridge)	8

syringae/

* Ps. prunicola is considered in this work to be synonymous with Ps. syringae (see Wilson, 1936).

SERIES II (contd.)

Specific name	Source	No. of strains
syringae	Dye (New Zealand)	2
atrofaciens	Dowson (Cambridge)	3
coronafaciens	" "	1
marginalis	" "	1
phaseolicola	" "	2
pisi	" "	2
rimaefaciens	" "	1
viridiflava	" "	1
--- **	Crosse (E. Malling)	6
--- **	Dye (New Zealand)	1

The species listed in Series I (A) are included in the above collection, making a total of 35 strains.

** These strains are unnamed but have been proved to be pathogenic to Prunus spp.

SERIES III.

The following organisms were isolated for this investigation from plants, some of which were diseased. Only a few of them have been proved to be pathogenic.

Plant source	No. of isolates
apricot	1
apple	1
barberry	2
bean	15
cauliflower	4

cherry/

SERIES III (contd.)

Plant source	No. of isolates
cherry	7
cherry laurel	4
cucumber	2
gladiolus	7
lilac	1
maple	3
mushroom	2
oats	7
peach	10
peas	13
plum	31
poplar	3
potato	1
raspberry	2
rhubarb	3
tomato	1

and the organisms in Series I (B), making a total of 123 strains.

SERIES IV.

The organisms of this group were isolated from sources not directly associated with plants, viz.:- 27 from soil, 17 from worm casts, with the addition of those of Series I (C), making a total of 46 strains.

The/

PART I

The water-soluble pigment.

The production of the pigment.

Introduction

The possession of this faculty by members of the genus Pseudomonas has been used for a considerable time as a distinctive and easily recognised diagnostic character. Despite its eminent suitability as a means of recognition of many members of the genus it is known that the character is not an invariable one. The early work of Charrin & Phisalix (1892) showed the loss of the chromogenic function of B. pyocyaneus, and achromogenic varients of the same organism were later described by Baerthlein (1918) and others. More recently, Burkholder (1930) confirmed that the ability of species of Pseudomonas to produce pigment was frequently lost in culture. Such cultures of his so-called "colourless group" did in fact produce pigment on certain media. An explanation of this inconstancy has not been made.

Efforts to provide suitable conditions for the production of pigments by pseudomonads have absorbed the attention of numerous workers. For example, Gessard (1890) was able to induce the formation of the fluorescent pigment alone, pyocyanin alone or the two pigments together. There followed many studies, cited by Turfitt (1936), on the problem of how to encourage or, at least, to explain the elaboration of these somewhat elusive compounds. A notable contribution was made by Benecke (1907) who showed that the essentials/

essentials for the production of fluorescent pigment in culture media were magnesium, phosphate, sulphate, and a very small amount of potassium with suitable sources of carbon and nitrogen. The later work of Georgia & Poe (1931, 1932) and Robinson (1932) confirmed most of Benecke's results. Robinson suggested that a high phosphate concentration inhibited pigment formation but, more recently, Burton, Campbell & Eagles (1948) noted that with higher concentrations of phosphate there was a proportional increase in the fluorescein produced. Pyocyanin production, on the other hand, decreased under these conditions.

The asparagine medium of Georgia & Poe (1931) was found by Turfitt to be satisfactory for the production of fluorescent pigment and the inhibition of pyocyanin. He noted that the asparagine could be replaced by ammonium nitrate. Of particular interest was his observation that if a trace of a heavy metal salt was added the pigment did not appear despite normal growth of the organisms. Impurities of tap water were said to be sufficient to prevent the development of any colour in the medium.

Clara (1934), who made a comparative study of the green fluorescent plant pathogens, described a medium well suited for the production of pigment. This again was very similar to that of Georgia & Poe and contained the same essential elements with asparagine as the source of carbon and nitrogen. One of the merits of this work is the/

the fact that a much wider series of Pseudomonas strains was studied by Clara than by previous workers.

The long search for pigment stimulants has led to the general finding that the most suitable media are those in which nitrogen is supplied by an inorganic salt or asparagine. Peptone media were studied by Georgia & Poe (1932) who found that their efficiency from the point of view of pigment production depended greatly on the source and purity of the many batches of peptone used. Certain of the green fluorescent types studied by Burkholder did not produce pigment on beef extract agar. Sodium lactate was used as a carbon source in a medium described by Seleen & Stark (1943) who found it to give results almost as good as Georgia & Poe's asparagine medium although a number of the cultures studied failed to produce pigment on it.

The observation by Turfitt of the effect of heavy metals on pigment formation was developed when Burton, Campbell & Eagles (1948) noted that, if the ferrous sulphate in their medium was omitted, a marked production of fluorescein by Ps. aeruginosa was obtained with no elaboration of pyocyanin. In the same year King, Campbell & Eagles (1948) gained more information of the effect of iron in media. They found that although iron was essential for growth, pigment formation occurred only if the concentration was kept at a low level, of the order of 0.05 p.p.m. Totter & Moseley (1953) found that at a pH of between 6 and 8.5/

8.5 the production of fluorescent pigment by Ps. aeruginosa was related inversely to the concentration of iron in the medium.

Despite the work described above no medium has been evolved which will invariably permit of pigment formation. Even slight changes in media were found by Ferguson Wood (1950) to cause changes in the results.

Experiment 1 A comparison of media.

It appears that the intensive study, which has been undertaken on the production of the fluorescent pigment by the pseudomonads, has yielded a considerable amount of information which has yet to be developed.

A preliminary experiment was carried out using a short series of representative strains (Series I, page 8) with the intention of comparing the pigment production on some of the more promising media, viz.,

A. Ordinary nutrient agar, (pH 7.0).

B. Georgia & Poe's (1931) asparagin.

$MgSO_4$	0.5 g.
K_2HPO_4	0.5 g.
asparagin	3.0 g.
dist. water	1000 ml.

C. Clara's (1934) asparagin No. 2.

$MgSO_4$	1.0 g.
K_2HPO_4	1.0 g.
asparagin	10.0 g.
dist. water	1000 ml.

D./

D. Stirling's (1951) ammonium lactate.

ammonium lactate (syrup)	10.0 ml.
glucose	2.0 g.
K_2HPO_4	1.0 g.
$MgSO_4.7H_2O$	0.2 g.
agar, washed shredded	15.0 g.
dist. water	1000 ml.

(pH 6)

Medium D was devised for use in counting Gram - organisms in bacteriological studies of silage. It was found that strains of Pseudomonas grew well and produced a strong pigmentation of the medium. The results of this comparison are presented in Table 1.

Results

Considerable variation was observed in the ability of the different media to show fluorescence. The ammonium lactate medium (D) was the most promising, while the results shown by media B and C were disappointingly poor. It was noticed that strains P_4 and P_5 showed no evidence of the pigment on any of the four selected media.

Experiment 2 · The use of ultraviolet light as an aid in the detection of the pigment.

In the comparison described above there was some uncertainty of the fluorescent pigment when it was present only in very small amounts. A suitable ultraviolet source was/

TABLE 1

A comparison of some media recommended for fluorescence production

Organism P-	Medium A	B	C	D
1	X	X-	-	XX
2	X	-	-	XX
3	XXX	XXX	XX	XX
4	-	-	-	-
5	-	-	-	-
6	-	X-	X-	XX
8	-	-	-	XX
9	-	-	-	XX
10	-	-	-	XX
11	XXX	X	X	XX
12	X	-	X	XXX

Key:-

Fluorescence was assessed by the naked eye appearance in north daylight.

- \- none detectable
- X- doubtful
- X weak
- XX moderate
- XXX strong

Media

A Ordinary nutrients agar (pH 7.0)
B Georgia & Poe's (1931) Asparagin.
C Clara's (1934) Asparagin No. 2.
D Stirling's (1951) Ammonium lactate.

was available* and all results relating to fluorescence in this study were subsequently obtained by examining the cultures with this aid.

Results

When exposed to an ultraviolet beam the cultures on the media noted in Table 1 were graded in the same relative order but the fluorescence where present was more intense. No increase in the numbers showing fluorescence was obtained.

Experiment 3 Fluorescence in litmus milk.

Of a series of 204 strains, 179 (87%) produced fluorescence when grown in litmus milk. Included among these strains were P_4 and P_5 which had failed to show pigment on the media previously mentioned. The same series showed similar results when grown in skimmed milk without the addition of an indicator. In this case it was slightly easier to detect fluorescence by naked eye but the aid of an u.v. lamp was still considered necessary in most cases.

The effect of different carbon sources.

The chemically defined media devised by many workers in the past have only been partially successful in overcoming the apparently capricious production of fluorescence. During the course of other investigations described elsewhere in/

* Technical lamp 11, Hanovia Ltd., Slough, England. (passing radiation of wavelength c. 3,660 Å.)

in this paper a large number of cultural tests were carried out using various carbon sources in a basal salts medium with inorganic nitrogen, e.g. that of Dowson (1949). While studying these cultures it was noticed that under u.v. light a large number showed a fluorescence which generally appeared more intense than that shown on organic media. The fluorescence in a selection of these has been noted in Table 2.

These results showed that fluorescence was produced by 48% or more of the strains in each carbon source utilised with the exception of sodium oxalate where the percentage was considerably less (column D).

Any medium selected for the determination of fluorescence should be able to support the active growth of all members of the genus and should permit the production of pigment to the point of easy recognition. With reference to Table 2 it is observed that with the exception of ethyl alcohol and sodium oxalate the carbon sources chosen all showed some promise of approaching these requirements.

It was decided to attempt the further improvement of media containing two of these carbon sources, namely, sodium gluconate and sodium succinate.

Experiment 4 The effect of lowering the concentration of heavy metals.

The first development was suggested by the work of Turfitt (1936), Burton, Campbell & Eagles (1948), and Totter

&/

TABLE 2

The incidence of fluorescence using various carbon sources in a basal salts medium

Carbon source	Number of strains tested	Number utilising C-source	Number showing fluorescence (in u.v.)	
	A	B	C	D (C as % B)
Na tartrate	204	155 (77%)	74	48
Na β -hydroxy butyrate	204	176 (88%)	133	73
Na fumarate	49	41 (84%)	22	54
Ethyl alcohol	212	66 (31%)	39	59
Glycerol	212	208 (98%)	147	71
Na succinate	212	201 (95%)	147	73
Na oxalate	212	85 (40%)	7	8
Na gluconate	204	204 (100%)	173	85

& Moseley (1953) concerning the effect of heavy metals and, in particular, that of iron in the media.

The iron content of the basal medium containing 0.5% sodium gluconate was lowered by an adsorption method suggested by Bard & Gunsalus (1950). 5 ml. of a 10% calcium chloride solution were added to each litre of the medium at a slightly alkaline pH value. The mixture was boiled for a few minutes, allowed to cool and filtered through paper. This treatment was repeated three or four times, replacing the original phosphate on each occasion. The final filtrate was adjusted to pH 7 and made up to the original volume with glass-distilled water. The medium was dispensed in 3 ml. quantities in tubes which had been cleaned and rinsed normally and not subjected to any special treatment to remove traces of heavy metals. It was considered sufficient and even advisable for the purpose to remove only a proportion of the iron present, otherwise a considerable restriction in the growth of the organisms might result.

The effect of this "iron-reduced" medium was studied using eleven different strains of Pseudomonas (Series I). For comparison the same organisms were grown in the untreated medium and also with the addition of iron citrate (final concentration 0.01%). The inoculum was in every case a small loopful from a 24-hour broth culture which had no visible effect on the opacity of the medium. The results of/

of this comparison are given in Table 3.

Results

Three noteworthy observations were made:-

(a) The amount of growth did not appear to bear a direct relationship to the pigment production.

(b) The fluorescence was produced more rapidly and strongly in the "iron-reduced" medium than in the others.

(c) The amount of growth (as indicated by opacity) was slightly less in the "iron-reduced" medium than in the others.

The relationship of the iron concentration and the production of fluorescent pigment was considered sufficiently important to merit further study.

Experiment 5 The effect of partial chelation of media.

Donald, Passey & Swaby (1952) tested a large number of methods and their modifications for removing trace metals from nutrient media. They described among others a modification of Waring & Werkman's (1942) method using 8-hydroxyquinoline (oxine) as a chelating compound. The use of oxine for this purpose was tested by Totter & Moseley (1953) who found that it failed to increase the pigment production by Pseudomonas strains. This unexpected result may have been due to the metallic content having been reduced too greatly or, alternatively, an excess of the chelating agent may have remained and rendered the medium unsuitable./

TABLE 3

The effect on the intensity of fluorescence of the iron content of a gluconate inorganic salts medium

Organism	Medium								
	A			B			C		
P	Growth 2 days	Fluor. 2 days	Fluor. 7 days	Growth 2 days	Fluor. 2 days	Fluor. 7 days	Growth 2 days	Fluor. 2 days	Fluor. 7 days
1	X	X	XX	XX	sl	X	XX	-	-
2	X	X	XX	XX	X	X	XX	-	-
3	X	XX	XX	XX	XX	XX	XX	sl	sl
4	X	X	XX	XX	X	XX	XX	-	sl
5	X	X	XX	XX	sl	sl	XX	-	sl
6	X	XX	XX	XX	sl	sl	XX	-	v.sl
8	X	X	XX	XX	X	X	XX	-	v.sl
9	X	X	XX	XX	X	X	XX	-	v.sl
10	X	X	XX	XX	X	X	XX	v.sl	v.sl
11	X	XX	XX	XX	XX	XX	XX	v.sl	v.sl
12	X	XX	XX	XX	X	X	XX	v.sl	v.sl

Key

Incubation at 27°C.

Fluorescence examined with the aid of u.v.

Medium

A ... "iron-reduced"
B ... untreated
C ... untreated + 0.01% iron citrate.

Growth

X ... moderate
XX ... heavy.

Fluorescence

- ... not detected
v.sl ... very slight
sl ... slight
X ... moderate
XX ... strong

unsuitable.

An attempt, however, was made to use oxine as a means of removing a proportion of the iron from the gluconate medium used in the previous experiment. The method adopted consisted in dissolving the constituents for 1 litre in 300 to 400 ml. of glass distilled water, adding 2 ml. of 0.5% w/v 8-hydroxyquinoline in redistilled chloroform (Waring & Werkman, 1942), and agitating vigourously for 2 min. in a separating funnel. The chloroform layer was allowed to settle out and was then removed. This process was repeated three further times with fresh chelating agent. Finally the solution was washed twice with 5 ml. quantities of redistilled chloroform to remove the excess oxine. After the removal of the visible traces of chloroform the solution was made up to 1 litre with glass-distilled water and dispensed in normally cleaned tubes and sterilised at 22 lb. pressure.

The medium after this treatment was compared with the normal medium using a series of 204 strains.

Results

The treatment with the oxine brought about a considerable improvement in the intensity of the fluorescence, (Table 4, A and B, and Figure 1, A and B) and in addition it permitted a larger number of cultures to show fluorescence, including all the mors-prunorum and syringae strains. A few of the other strains tested showed only a weak to moderate/

TABLE 4

The influence on fluorescence of modifications of the gluconate medium

(see also Figure)

Medium	T_F		FF		F		F_S	
	No.	%	No.	%	No.	%	No.	%
A	173	85	34	17	67	33	72	35
B	199	98	133	65	61	30	5	3
C	188	92	95	46	32	16	61	30
D	182	89	125	61	24	12	33	16
E	189	92	140	69	17	8	32	16
F	199	98	179	88	14	7	6	3

Key

The percentages are based on the number of strains tested, i.e. 204.

T_F The total number of strains showing fluorescence.

FF The number showing a strong fluorescence.

F The number showing a moderate fluorescence.

F_S The number showing a slight fluorescence.

The media were composed of inorganic salts with the following additions:-

A, B - gluconate (0.5%)

C, D - gluconate (0.5%) and asparagine (0.1%)

E gluconate (0.5%) and asparagine (0.1%) (incubated for 14 days)

F gluconate (0.5%) and casein hydrolysate (0.1%)

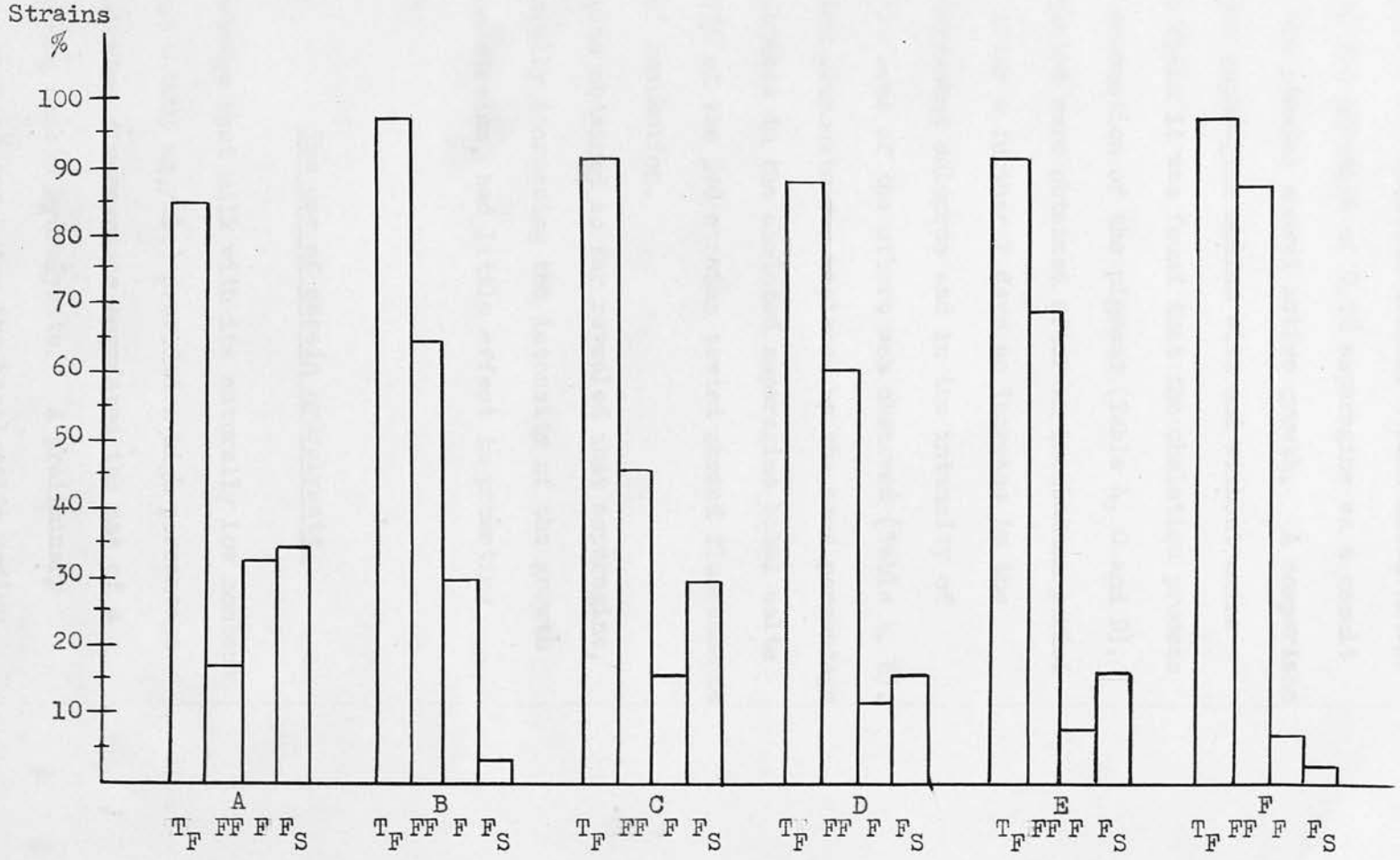
All of the above media, except A and C, were oxine-treated.

Except in the case of medium E the cultures were incubated for 7 days at 27°C.

FIGURE 1

THE INCIDENCE OF FLUORESCENCE IN MODIFICATIONS OF THE GLUCONATE MEDIUM

(A diagrammatic representation of the results presented in Table 4)



Key: as for Table 4.

moderate growth on the chelated medium. This disadvantage was avoided by the addition of 0.1% asparagine as a result of which all the strains showed active growth. A comparison was made of the asparagine medium with and without oxine treatment and again it was found that the chelation process favoured the production of the pigment (Table 4, C and D). The figures quoted were obtained after an incubation period of 7 days. After a further 7 days an increase in the number of fluorescent cultures and in the intensity of fluorescence of some of the others was observed (Table 4, E). When the sodium gluconate was replaced by the same percentage of sodium succinate in the chelated asparagine basal salts medium only 67% of the 210 strains tested showed fluorescence after 14 days' incubation.

The results obtained so far revealed that asparagine, although generally increasing the intensity of the growth of the various strains, had little effect in promoting fluorescence.

Experiment 6 The use of casein hydrolysate.

The knowledge that milk with its naturally low content of iron (about 0.0005 mg./ml.) provided a high percentage of cultures showing fluorescence suggested the use of a medium containing casein hydrolysate. A preliminary experiment was carried out using the basal salts medium with the addition of 0.1% casein hydrolysate and 0.5% sodium/

sodium gluconate. The medium was not chelated. The results obtained with the 52 strains tested were recorded after 7 days' incubation at 27°C. and are given in Table 5.

Results

Strains which had found difficulty in growing in media A and B now grew easily. This and the fact that a very high percentage of cultures were fluorescent suggested that efforts should be made to improve the medium.

Experiment 7 The effect of the partial chelation of a casein hydrolysate medium.

When the casein hydrolysate-gluconate medium was chelated by the oxine method described above and then tested with a series of 204 organisms a high proportion of the cultures produced a strong fluorescence (Table 4, F; and Figure 1, F). The incidence of strong fluorescence was greater in this case than with any other of the media so far examined. The total number of cultures, showing at least some degree of fluorescence, however, was the same as that obtained using the chelated gluconate medium (B).

Conclusion

From this series of trials it was concluded that for routine purposes the use of the chelated gluconate medium (B) is satisfactory for the detection of fluorescence if readings are made with the aid of an ultraviolet lamp. This medium has the advantage of being completely non-fluorescent when sterile/

sterile, a useful property when weak fluorescence must be recognised.

TABLE 5

The incidence of fluorescence in a gluconate (0.5%) casein hydrolysate (0.1%) medium

	No.	%
Strains tested	52	100
Strains fluorescent	49	92
Strong fluorescence	37	70
Moderate fluorescence	8	15
Slight fluorescence	4	7

The effect of metals on the appearance of the pigment.

Introduction

The foregoing experiments showed that treatment of media with a chelating agent greatly increased the production of the fluorescent pigment of these organisms. Some explanation of the fact was obviously required. Two possibilities were considered:-

(a) A metal (e.g. iron) might form a non-fluorescent compound or complex with the pigment. If this were so it might be possible to remove the metal and reproduce the fluorescence.

(b) A restriction in the formation of an enzyme system of which iron is an essential component might lead to an accumulation of the pigment. In that case the pigment could be considered as an intermediate product which, if sufficient iron were present, would be further metabolised to a non-fluorescent compound. With regard to these possibilities it is interesting to note that of several hundred examinations of plant lesions caused by Pseudomonas spp. none ever showed a typical fluorescence under u.v. light.

Experiment 8 The isolation of the pigment.

In order to explore some of the relevant properties of the pigment an attempt was made to obtain it in a reasonably pure/

pure and concentrated form. Attempts to isolate the pigment and determine its chemical nature have been made by Giral (1936), Turfitt (1937) and Turfreijer, Wibaut & Boltjes (1938). Their results were not altogether in agreement and the chemistry of the pigment is still somewhat uncertain. By modifying the isolation procedure adopted by Turfitt a strongly fluorescent solid was obtained which, although not chemically pure, was suitable for the tests envisaged.

The preparation was carried out as follows:-
The oxine-treated gluconate medium (B) was prepared and 12 litres were distributed in four 5-litre flasks fitted with glass tubing for aeration purposes. These were sterilised and inoculated with a strain (P_3), known to be active in pigment production. Air, sterilised by passage through cotton wool filters was continuously bubbled through the flasks. The apparatus was sited in a greenhouse where the temperature ranged between 12°C and 23°C. during the incubation period of 7 days. A strong yellow green colour was produced and by 7 days it had apparently reached its maximum intensity. The cultures were then acidified with dilute acid (4 ml. 2N-H_2SO_4/100 ml.) and common salt was added (20 g./100 ml.) to precipitate the cells and proteinaceous materials from the liquid. After passing through coarse filter paper the resulting clear yellowish solution was mixed with freshly activated sugar charcoal (1 g./100 ml.) and/

and heated to almost boiling point for a few minutes. After standing overnight the suspension was filtered through paper pulp. The filtrate was tested under u.v. light when acid and also when alkaline and found to be non-fluorescent. The residue of charcoal was washed with dilute hydrochloric acid until phosphate-free and thereafter washed free of chlorides with distilled water.

The washed residue was then treated with acetic acid (glacial) until the washings were no longer fluorescent. The eluted pigment solution was reduced to small bulk by vacuum distillation at a temperature not exceeding 55°C. and finally dried in an incubator at 37°C. The resulting product was a brown solid which dissolved readily in water and was intensely fluorescent. The total yield (approx. 0.2 g.) was dissolved in 100 ml. of glass distilled water. This solution was used in the investigations described below.

Experiment 9 The effects of iron and copper salts.

The first of these experiments showed the effect of adding acid or alkaline solutions of ferrous sulphate (0.1%) in equal volume to the pigment solution. The fluorescent pigment uranin was subjected to the same treatments for comparison.

Results

It was observed that the iron salt distinctly quenched the/

the fluorescence when the solution was alkaline (Table 6). The fluorescence was restored on adding acid. These changes could be repeated indefinitely. The effects were similar with the uranin solution. The iron salt in acid solution reduced the intensity of the fluorescence only slightly. When copper sulphate was used in place of iron sulphate the results were identical.

Experiment 10 Further effects of an iron salt.

A further demonstration of this effect was shown by the following simple experiment. A 2 ml. quantity of a 0.1% solution of $FeSO_4.7H_2O$ was added to and mixed with 50 ml. water agar and poured into Petri dishes which were tilted slightly to provide an uneven layer. This was allowed to set and water agar containing the extracted pigment was poured on top of the first layer. Two such plates were prepared. In one case (a) the ferrous sulphate solution was made slightly acid and in the other (b) the solution was slightly alkaline (Figure 2). These plates were exposed to u.v. light first on one surface and then on the other and the resulting appearances noted.

Results

The pigment-agar surface was in both cases intensely fluorescent. On examination through the iron salt layer the pigment showed a slightly decreased intensity of fluorescence/

TABLE 6

The effect of acid and alkali on the quenching of fluorescence by ferrous sulphate

Treatment	Fluorescent culture extract (appearance under u.v.)	Uranin (appearance under u.v.)
a) neutral	yellow-green (XXX)	yellow-green (XXX)
b) acid	white-blue (XXX)	yellow green (XXX)
c) alkaline	green (XXX)	yellow-green (XXX)
d) acid & Fe	white-blue (XX)	yellow-green (XX)
e) alkaline & Fe	green (X)	yellow-green (XX)
f) as e) but finally acid	reappearance of white-blue (XX)	yellow-green (XX)

Key

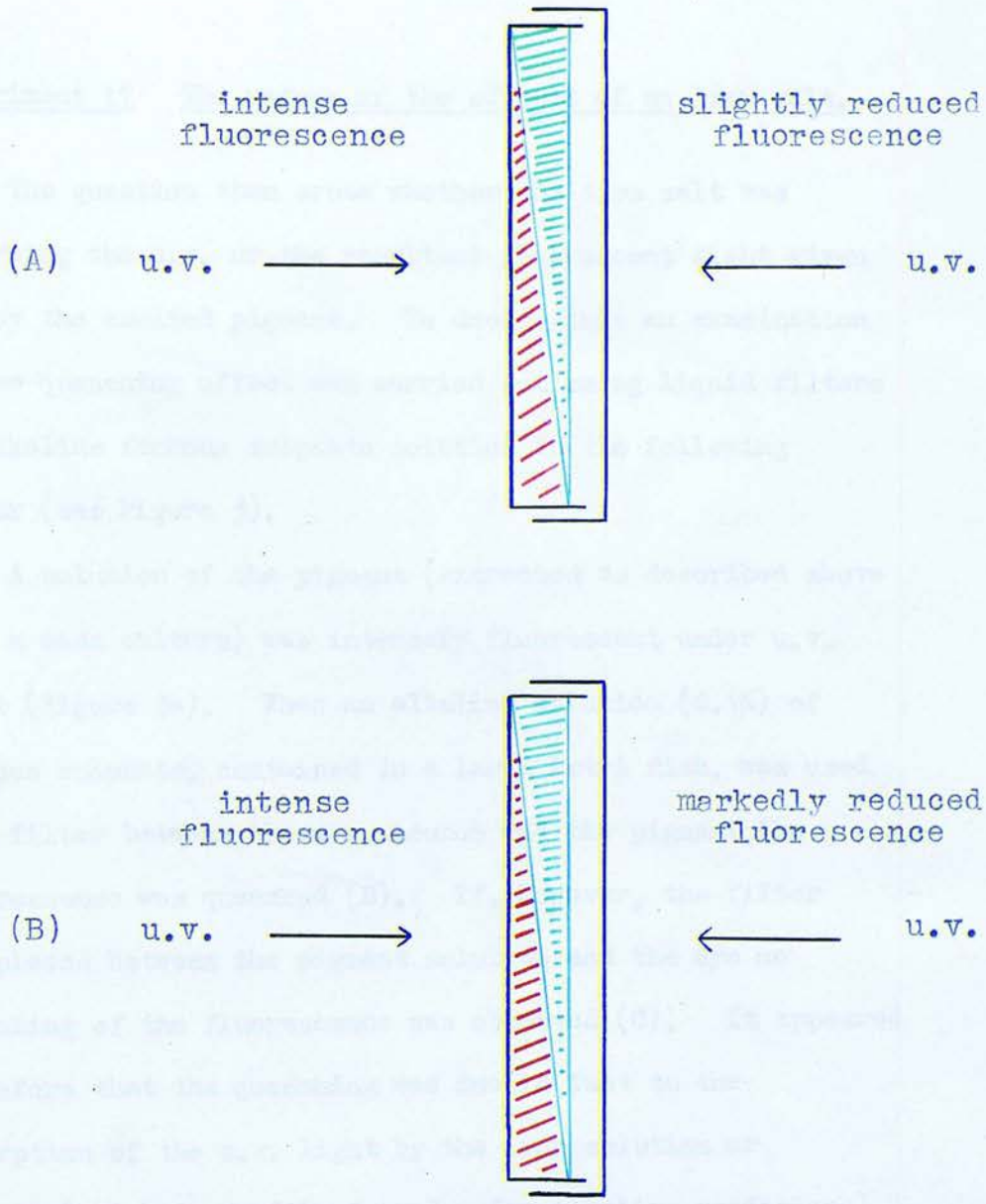
XXX ... strong fluorescence

XX ... Moderate fluorescence

X ... weak fluorescence

Figure 2.

The demonstration of the effect of acid and alkali on the quenching of fluorescence by ferrous sulphate.



Key:

(A) Ferrous sulphate agar (acid).
(B) " " " (alkaline).

fluorescence where the layer was acid but little or no fluorescence where the layer was alkaline.

Experiment 11 The nature of the effects of an iron salt.

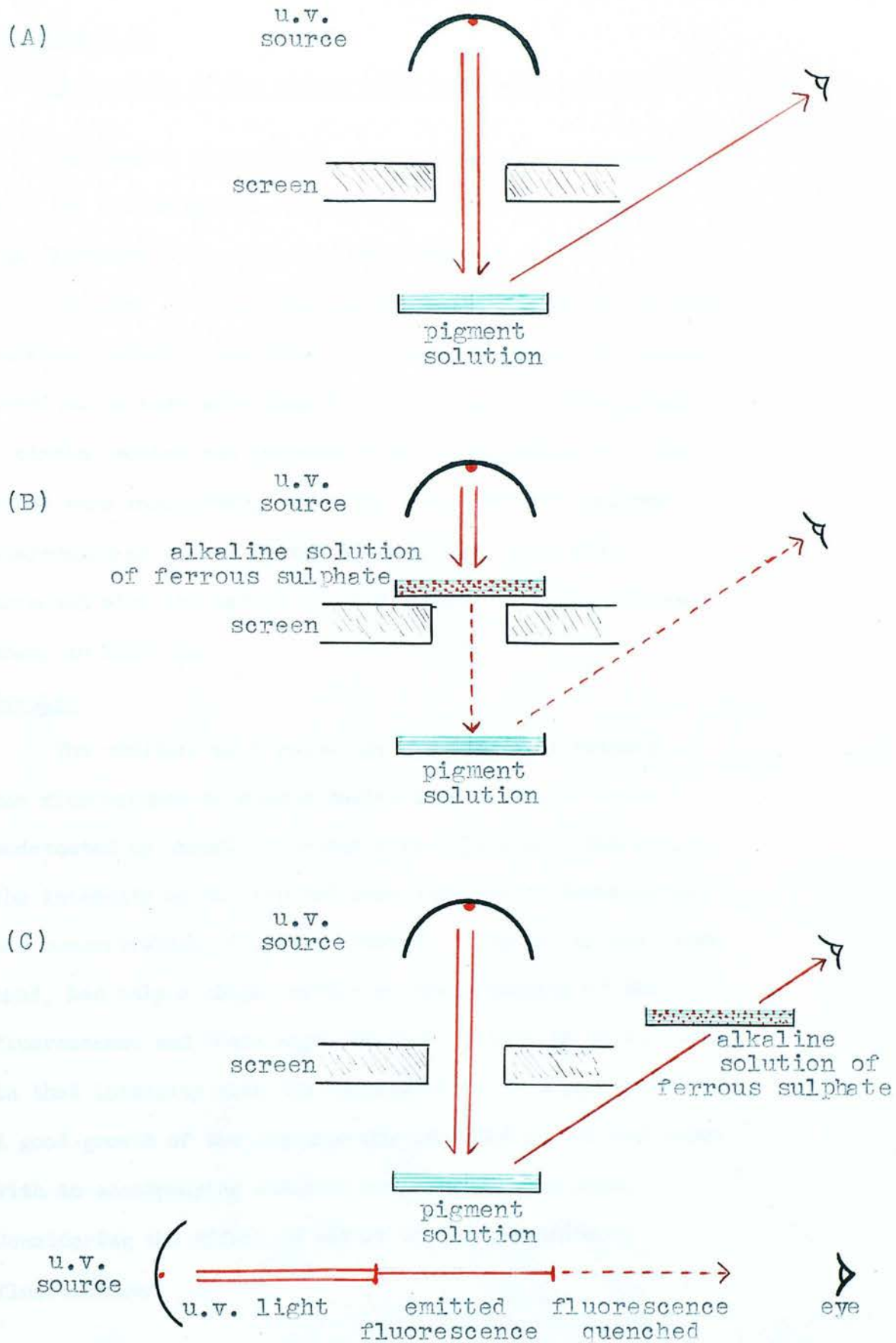
The question then arose whether the iron salt was absorbing the u.v. or the resultant fluorescent light given off by the excited pigment. To decide this an examination of the quenching effect was carried out using liquid filters of alkaline ferrous sulphate solution in the following manner (see Figure 3).

A solution of the pigment (extracted as described above from a mass culture) was intensely fluorescent under u.v. light (Figure 3a). When an alkaline solution (0.1%) of ferrous sulphate, contained in a large Petri dish, was used as a filter between the u.v. source and the pigment the fluorescence was quenched (B). If, however, the filter was placed between the pigment solution and the eye no quenching of the fluorescence was observed (C). It appeared therefore that the quenching was due in fact to the absorption of the u.v. light by the iron solution or colloid (which is readily formed under alkaline conditions) rather than to any chemical affinity of the metal for the pigment. Only the salts of heavy metals were found to produce this effect.

Experiment 12/

Figure 3.

The demonstration of the absorption of u.v. light by an alkaline solution of ferrous sulphate.



Experiment 12

The nature of the effect of an iron salt (contd.)

The second possibility, that iron might be associated with the enzyme system responsible for the production of the fluorescent pigment, was then studied.

To tubes of oxine-treated gluconate medium (B) ferrous sulphate solution was added to provide a series of concentrations of that salt from 5 to 25 p.p.m. (as $FeSO_4.7H_2O$). A similar series was prepared with copper sulphate. The tubes were inoculated, each with a loopful of a uniform s suspension of strain P3 and after 48 hrs. they were examined with the aid of the u.v. lamp. The results are shown in Table 7.

Results

The addition of 5 p.p.m. of the iron salt reduced the fluorescence to such a degree that it would remain undetected by visual observation in daylight. Under u.v. the intensity of the fluorescence appeared to diminish as the concentration of iron increased. Copper, on the other hand, had only a slight effect on the intensity of the fluorescence and there appeared to be little or no variation in that intensity with the concentration of copper present. A good growth of the organism was produced in all the tubes with an accompanying alkaline reaction in each case. Considering the effect of alkali on the quenching of fluorescence/

TABLE 7

The effect on fluorescent pigment production of the addition of ferrous sulphate and of cupric sulphate to an iron-reduced culture medium

Culture no.	Concentration (p.p.m.)	Relative intensity of fluorescence (u.v.)
1	($FeSO_4.7H_2O$)	
1	5	XX
2	10	XX
3	15	X
4	20	X/-
5	25	X/-
6	nil	XXX
	($CuSO_4.7H_2O$)	
1	5	XX
2	10	XX
3	15	XX
4	20	XX
5	25	XX
6	nil	XXX

Key

XXX ... strong fluorescence
XX ... moderate fluorescence
X ... weak fluorescence
X/- ... doubtful fluorescence

fluorescence in the presence of an iron salt (page 27) an attempt was made to increase the intensity of the pigment by acidifying the cultures but this proved unsuccessful.

It appeared therefore that there was no masking of the fluorescence by the traces of metal employed in this particular experiment. This suggests that iron effects either the production or a subsequent destruction of the pigment.

Conclusions (Experiments 1 - 12)

Media recommended in the literature for the demonstration of fluorescence have given unsatisfactory results with a wide range of isolates.

Traces of iron in media were found to diminish or abolish the fluorescence. The nature of the effect was not fully determined.

A gluconate inorganic salts medium after treatment with 8-hydroxyquinoline was found to be the most satisfactory of the media examined for the demonstration of pigment production by strains of Pseudomonas, including Ps. morsprunorum and Ps. syringae.

The activities of the organisms on various carbohydrates.

Introduction

Records of work carried out on the carbohydrate metabolism of the pseudomonads were few in number until recent years when interest was aroused in the enzyme systems involved. Most of this later work, reviewed by Campbell (1954), has been performed on one or other of the two main species, Ps. aeruginosa and Ps. fluorescens using, in most cases, glucose or its derivatives as the metabolites. Whether the results obtained are applicable to other species is a matter still to be determined.

In the literature there are numerous records of acid formation from sugars by different species. De Bord (1923) reported that in 2% peptone broth containing 1% glucose all the sugar could be utilised without producing at any time an acid reaction. Sears (1916) had earlier demonstrated an active metabolism of nitrogenous substances by Ps. aeruginosa even in the presence of glucose, and later Sears & Gourlay (1928) showed that the acid products of glucose decomposition were neutralised by the products of the nitrogen metabolism. This masking effect was removed by keeping the nitrogen content of the medium low. When they used other sugars in place of glucose an acid reaction was not produced even when the nitrogen content was low despite the fact that the sugars tested were said to be capable of being/

being utilised by the organism. Lacey (1932), in a comparison of members of the genus associated with plant diseases, found that 90% showed acid production from glucose, 68% from sucrose but none from lactose. The acid production from sucrose was said to be very variable. A similar study by Clara (1934) indicated that the production of acid from glucose and sucrose but not from lactose were important characters of these bacteria. All the monosaccharides tested except rhamnose were fermented by all the organisms in the survey which included Ps. aeruginosa and Ps. fluorescens. None of them was recorded as being capable of fermenting maltose or lactose.

Sandiford (1937), when comparing Ps. aeruginosa and Ps. fluorescens, stated that acid was formed from glucose by the latter but not by the former organism. Elrod & Braun (1942) found that acid was produced from glucose but not from lactose or sucrose by Ps. aeruginosa. Later Salvin & Lewis (1946) used the same synthetic medium as Elrod & Braun to study a group of isolates capable of producing pyocyanin and confirmed the latters' results for glucose, lactose and sucrose. According to Erikson (1945), who compared Ps. mors-prunorum with related phytopathogenic bacteria, an acid reaction was obtained in a synthetic medium for all the mono- and tri-saccharides on which growth was maintained. In the case of the disaccharides acid production was demonstrated from sucrose but not from lactose or maltose. Variable results were reported by

Gaby/

Gaby (1946) using Ps. aeruginosa. Liu (1952) noted that the total amount of alkali produced by Ps. aeruginosa was only a little more than that produced under similar conditions by Escherichia coli or Aerobacter aerogenes and that the failure of the pseudomonads to show acid production was due to the small amount of acid produced from carbohydrates. Burkholder (1930) made little or no reference to the nature of the medium from which the acid reactions, he mentions, are produced.

Experiment 13

Acid formation from glucose, lactose and sucrose.

Experiments were designed to show whether or not acid formation from glucose, lactose and sucrose would provide a differentiation of the organisms being examined. In view of the earlier work showing that pseudomonads generally produce only small amounts of acid, and the indications that acid formation may be detectable in one medium and not in another, particular attention was given to the sources of nitrogen and the sugar-nitrogen ratio. Three sets of media were prepared.

The first (A) contained glucose, lactose or sucrose at concentration of 0.5, 1.5 and 5.0% with peptone at concentrations of 0.1 to 1.0%, giving a series of sugar-peptone ratios ranging from 1:2 to 50:1. The media were adjusted/

adjusted to pH 7.0 before sterilisation.

In the second medium (B) the same sugars were incorporated at concentrations of 0.5, 1.5 and 5.0% in an ammonium salt solution of the following composition:-

$(NH_4)H_2PO_4$	0.1 g.
KCl	0.02 g.
$MgSO_4$	0.02 g.
Dist. water	100 ml.

The three sugars were used in the third medium (C) in the same concentrations. The medium was composed of:-

KNO_3	0.1 g.
KH_2PO_4	0.1 g.
$MgSO_4$	0.02 g.
NaCl	0.02 g.
Dist. water	100 ml.

The above media were tubed and sterilised under pressure with and without agar and with brom cresol purple as pH indicator. The tubes were inoculated with the strains of Series I (see Introduction, page 8) and incubated at 22°C.. Examinations were made at frequent intervals up to 54 days and the indicator changes observed.

Results

Glucose. The eleven strains all produced similar effects on the glucose-peptone medium (A). Acid was produced at all ratios within 48 hrs. In some cases a reversion to a higher pH occurred which appeared to be a function of the sugar/

TABLE 8

The effects of different concentrations of GLUCOSE and PEPTONE on acid production

Sugar conc. %		0.5	0.5	0.5	0.5	1.5	1.5	1.5	1.5	5.0	5.0	5.0	5.0
Peptone conc. %		1.0	0.5	0.2	0.1	1.0	0.5	0.2	0.1	1.0	0.5	0.2	0.1
S/P ratio		1:2	1:1	2.5:1	5:1	1.5:1	3:1	7.5:1	15:1	5:1	10:1	25:1	50:1
Strain P	1	A2.R5	A2.R13	A2.R13	A2	A2	A2	A2	A2	A2	A2	A2	A2
	2	A2.R6	A2.R13	A2.R6	A2	A2	A2	A2	A2	A2	A2	A2	A2
	3	A1.R6	A1.R13	A1.R6	A1	A1	A1	A1	A1	A1	A1	A1	A1
	4	A2.R6	A2.R13	A2.R13	A2	A2	A2	A2	A2	A2	A2	A2	A2
	5	A2.R13	A2.R26	A2.R26	A2	A2	A2	A2	A2	A2	A2	A2	A2
	6	A2.R13	A2.R13	A2.R13	A2	A2	A2	A2	A2	A2	A2	A2	A2
	8	A2.R13	A2.R13	A2.R13	A1	A1	A1	A1	A1	A1	A1	A1	A1
	9	A2.R13	A1	A1	A1	A1	A1	A1	A1	A1	A1	A1	A1
	10	A2.R13	A1	A1	A1	A1	A1	A1	A1	A1	A1	A1	A1
	11	A2.R13	A1.R13	A1.R13	A1	A1	A1	A1	A1	A1	A1	A1	A1
	12	A1	A1	A1	A1	A1	A1	A1	A1	A1	A1	A1	A1

Key

A ... acid (B.C.P.)
R ... reversion

The figures refer to the number of days' incubation.

TABLE 9

The effects of different concentrations of LACTOSE and PEPTONE on acid production.

Sugar conc. %	0.5	0.5	0.5	0.5	1.5	1.5	1.5	1.5	5.0	5.0	5.0	5.0
Peptone conc.%	1.0	0.5	0.2	0.1	1.0	0.5	0.2	0.1	1.0	0.5	0.2	0.1
S/P ratio	1:2	1:1	2.5:1	5:1	1.5:1	3:1	7.5:1	15:1	5:1	10:1	25:1	50:1
Strain P 1	N	N	N	N	N	N	A26	A26	A54	A26	A26	A26
2	N	N	N	N	N	N	A26	A26	A54	A26	A26	A26
3	N	N	A26	A26	N	A26	A6	A6	A6	A6	A2	A2
4	N	N	N	A26	N	A26	A26	A26	A26	A26	A26	A26
5	N	N	N	A54	N	A54	A54	A54	A54	A26	A26	A26
6	N	N	N	A26	N	N	A13	A13	A13	A6	A6	A6
8	N	N	A26	A13	A54	A26	A6	A6	A13	A6	A6	A6
9	N	N	A54	A26	N	A26	A13	A13	A26	A13	A2	A2
10	N	N	N	A26	N	N	A26	A26	A26	A26	A6	A6
11	N	N	A54	A26	N	A54	A13	A13	A26	A26	A6	A6
12	N	N	A54	A13	N	A54	A6	A6	A13	A6	A6	A6

Key

A ... acid (B.C.P.)
N ... no pH change recorded

The figures refer to the number of days' incubation.

TABLE 10

The effects of different concentrations of SUCROSE and PEPTONE

on acid production

Sugar conc. %	0.5	0.5	0.5	0.5	1.5	1.5	1.5	1.5	5.0	5.0	5.0	5.0
Peptone conc.%	1.0	0.5	0.2	0.1	1.0	0.5	0.2	0.1	1.0	0.5	0.2	0.1
S/P ratio	1:2	1:1	2.5:1	5:1	1.5:1	3:1	7.5:1	15:1	5:1	10:1	25:1	50:1
Strain P 1	A2.R13	A2.R13	A2.R13	A2.R13	A2	A2	A2	A2	A2	A2	A2	A2
2	A6.R13	A6.R13	A2.R13	A2.R13	A2.R13	A2	A2	A2	A2	A2	A2	A2
3	N	N	A13	A13	A13	A13	A6	A2	A2	A2	A2	A2
4	N	N	A6.R13	A2.R13	A2.R13	A2	A2	A2	A2	A2	A2	A2
5	N	N	N	N	N	N	N	N	N	N	N	N
6	A2.R13	A2.R13	A2.R13	A2	A2	A2	A2	A2	A2	A2	A2	A2
8	A6.R26	A6.R26	A6.R26	A6.R26	A6	A6	A6	A2	A2	A2	A2	A2
9	A6.R26	A6.R26	A2.R26	A2.R26	A2.R26	A2.R54	A2	A2	A2	A2	A2	A2
10	N	N	N	N	N	N	N	N	N	N	N	N
11	N	N	N	N	N	N	N	N	N	N	N	N
12	N	N	N	N	N	N	N	N	N	N	N	N

Key

A ... acid (B.C.P.)
R ... reversion
N ... no pH change recorded

The figures refer to the number of days' incubation.

TABLE 11

The effects of different concentrations of sugars on acid production in an ammonium salt medium

Strain	Glucose %			Lactose %			Sucrose %		
	0.5	1.5	5.0	0.5	1.5	5.0	0.5	1.5	5.0
P 1	A2	A2	A2	N	N	N	A2	A2	A2
2	A4	A2	A2	N	N	N	A4	A4	A2
3	A1	A1	A1	N	N	N	A6	A6	A1
4	A2	A2	A2	N	N	N	A2	A2	A2
5	A2	A2	A2	N	N	N	N	N	A2
6	A2	A2	A2	N	N	N	A2	A2	A2
8	A1	A1	A1	N	N	N	A6	A4	A1
9	A1	A1	A1	N	N	N	A6	A4	A1
10	A1	A1	A1	N	N	N	N	A15	A1
11	A2	A2	A2	N	N	N	N	N	A2
12	A2	A1	A1	N	N	N	N	N	A2

Key

A ... acid (B.C.P.)
R ... reversion
N ... no pH change recorded

The figures refer to the number of days' incubation.

TABLE 12

The effects of different concentrations of sugars on acid production in a nitrate salt medium

Strain	Glucose % 0.5	1.5	5.0	Lactose % 0.5	1.5	5.0	Sucrose % 0.5	1.5	5.0
P 1	A7	A7	A7	N	N	N	A7	A7	A7
2	A7	A7	A7	N	N	N	A7	A7	A7
3	A7	A7	A7	N	N	N	N	A49	A17
4	A7	A7	A7	N	N	N	N	A17	A17
5	N	A49	A49	N	N	N	N	N	N
6	A17	A7	A7	N	N	N	N	A7	A7
8	A7	A7	A7	N	N	N	N	A17	A17
9	A3	A3	A3	N	N	N	N	A17	A7
10	A17	A3	A3	N	N	N	N	N	N
11	A7	A3	A3	N	N	N	N	N	N
12	A3	A3	A3	N	N	N	N	N	N

Key

A ... acid (B.C.P.)
R ... reversion.
N ... no pH change recorded

The figures refer to the number of days' incubation.

sugar concentration rather than that of the sugar-peptone ratio. For example, reversions occurred frequently at 0.5% glucose but not at 1.5% although in both cases the sugar-peptone ratio was approximately the same (2.5:1 and 3:1). In addition it was noted that there were no reversions at any ratio where the sugar concentration exceeded 0.5% (Table 8). Ps. mors-prunorum (P_6) showed a slower reversion than Ps. syringae (P_1) at the lowest glucose-peptone ratio tested.

With the basal medium (B) all eleven organisms again produced acid from glucose within 48 hrs. but no reversions occurred at any of the concentrations studied (Table 11).

In the case of the nitrate-glucose medium (C) the organisms all produced acid but here the pH change was slower (with an average of 7 days). Three strains in the series (P9, P11 and P12) gave an acid reaction within three days (Table 12).

No important difference was observed between Ps. mors-prunorum (P_6) and Ps. syringae (P_1) in the glucose media B and C.

Lactose. In the peptone medium (A) the strains tested showed little activity towards lactose. At the lower sugar-peptone ratios a pH change was only detectable in a few cases and only after periods within the range of 26 to 54 days. At the higher ratios (7.5:1 and above) some of the test/

test strains including Ps. mors-prunorum produced a pH change in the shorter time of 6 to 13 days and all showed an acid reaction by 54 days (Table 9).

No acid reactions were detected in the lactose media (B) (Table 11), and the same result was noted with lactose media (C) (Table 12).

Sucrose. With medium (A) four of the eleven strains tested failed to show an acid reaction at any time during the period of incubation. At low sucrose-peptone ratios reversion frequently followed acid production within 26 days. Two strains (P_3 and P_4) produced acid only where the sucrose-peptone ratio was high (Table 10).

In medium (B) the sucrose was utilised by all eleven strains and acid was detected in all cases when the sugar concentration was at the high level of 5%. The four strains which failed to produce detectable acid in medium (A) again distinguished themselves from the others by failing to show an acid reaction at the lower ratios, (Table 11).

When the series was grown in the sucrose-nitrate medium (C) the same four strains noted above showed no acid reaction at any of the sugar concentrations used while a number of others produced acid only at the 1.5% and 5% levels. Only two strains showed the presence of acid at all three concentrations (Table 12).

In no case did the sucrose media effectively distinguish

Ps./

Ps. mors-prunorum from Ps. syringae.

Discussion

The results described above suggest that although the strains tested all appear to cause a similar breakdown of glucose the actual reaction observed depends largely on the concentration of the sugar and the nature and concentration of the nitrogen source present in the medium. The reactions obtained thus provided no obvious means of differentiation. Lactose was not a readily available energy source for the organisms but it was noted that the dissimilation of the sugar could be demonstrated when it was present in a high concentration in a peptone medium. The only apparent variation was the time taken by the various strains to produce an acid reaction.

The pseudomonads have been described generally in Bergey's Manual of Determinative Bacteriology (1948) as being 'non-fermenters' of lactose. However, Wormald (1932) in a description of Ps. mors-prunorum found that some strains of that species produced an alkaline followed by an acid reaction in a Difco nutrient broth containing 1% lactose and litmus. A method was subsequently evolved which was said to distinguish between the two closely related organisms Ps. mors-prunorum and Ps. prunicola. The eventual acid reaction of the former and the alkaline reaction of the latter served to differentiate the two organisms. It would appear/

appear from the above results that such an acid reaction is not necessarily confined to Ps. mors-prunorum. A brief investigation confirmed this. It was found that, of a selection of 140 Pseudomonas strains, including saprophytes, a large proportion were capable of producing a reaction similar to that of Ps. mors-prunorum in Wormald's medium and that the number of organisms giving a positive result depended to a considerable degree on the period of incubation (Table 13).

In addition to the six strains of Ps. mors-prunorum tested, five out of eleven strains of Ps. syringae showed an alkaline followed by an acid reaction.

The results obtained with sucrose were more interesting. Two groups could be demonstrated on the basis of acid production particularly in the peptone medium (A) where the sugar-peptone ratio was high. According to Clara (1934) the ability or inability of the pseudomonads to ferment sucrose divides them into two general sub-groups. These sub-groups were said to provide a natural division of the genus into those which were pathogenic to plants and those which were not. The latter group contained, in addition, some so-called 'weak parasites' and Ps. fluorescens. Previously, however, Lacey (1931, 1932) considered that the fermentation of sucrose was of little value in differentiation owing to an observed variability in acid production. More recently, Burkholder & Starr (1948) in agreement with Clara suggested/

TABLE 13

Acid production from 'purple lactose agar' *

	No.	%
Organisms tested	140	100
Organisms showing acid reaction after 4 days	36	25
Organisms showing acid reaction after 7 days	59	42
Organisms showing acid reaction after 21 days	102	72

* 2% lactose in nutrient agar with brom thymol blue as indicator (see Wormald, 1932).

suggested that such a division on the basis of sucrose dissimilation was both reliable and easily determined. The above experiments provided little further information as to the validity of such a grouping but the position was considered worthy of more detailed study.

Experiment 14

The effect of different nitrogen sources on acid production from sucrose.

A number of strains (204) were inoculated into three media each containing 1% sucrose and with brom cresol purple.

Medium (a) inorganic as (B) described above.

" (b) peptone (Evans) 0.5%

" (c) tryptone (Difco) 0.5%

After 7 days' incubation at 27°C. those cultures showing an acid reaction were noted. The results were analysed to show the reactions of strains originating from plants (regardless of pathogenicity), authentic pathogenic strains, and strains isolated from sources other than plants (Table 14).

Results

A notable feature of the results was that the number of strains which showed an acid reaction was greater in the synthetic or tryptone media than in peptone. No attempt was made to explain the difference. The use of the ammonium salt medium provided no obvious division in terms of/

TABLE 14

Acid production from 1% sucrose

(in peptone, tryptone and ammonium salt media with brom cresol purple as indicator)

A.	Isolations from all sources.		%
	Organisms tested	204	100
	Organisms showing acid in synthetic medium	118	58
	Organisms showing acid in peptone medium	74	36
	Organisms showing acid in tryptone medium	120	59
	Organisms not utilising sucrose in synthetic medium	17	8
B.	Isolation from plants (including authentic pathogens)		
	Organisms tested	158	100
	Organisms showing acid in synthetic medium	91	58
	Organisms showing acid in peptone medium	73	46
	Organisms showing acid in tryptone medium	106	68
C.	Isolations from sources other than plants.		
	Organisms tested	46	100
	Organisms showing acid in synthetic medium	27	61
	Organisms showing acid in peptone medium	1	2
	Organisms showing acid in tryptone medium	14	30
D.	Authentic pathogens.		
	Organisms tested	35	100
	Organisms showing acid in synthetic medium	31	86
	Organisms showing acid in peptone medium	31	86
	Organisms showing acid in tryptone medium	33	91

of habitat on the basis of acid production. In fact, the proportion of organisms showing acid were roughly of the same order whether they originated from plants or from sources other than plants, (Table 14, B and C). It was noted that a small group failed to grow in the synthetic medium.

The six mors-prunorum strains showed an acid reaction on all three media. Of the eleven syringae strains tested, all produced acid with the exception of two which produced no growth in the inorganic medium.

With the peptone medium one feature appeared worthy of some note. Of the 46 strains isolated from sources other than plants only one showed acid. (This exception was one of the series isolated from earthworm casts.) A large proportion of the isolates from plants showed an acid reaction.

In general, therefore, it appeared that the observed formation of acid by these bacteria depended to a large extent on the medium employed and even on the brand of peptone used. All the Ps. mors-prunorum and all but two of the Ps. syringae strains tested produced acid on each of the media. The remainder, it was noted, did not utilise sucrose as a sole source of carbon. This ability to utilise sucrose as a sole carbon source, appears to be of more importance than acid production.

Experiment 15/

Experiment 15 Copper-reduction by sucrose cultures.

It was found that a proportion of the cultures from Experiment 14 (all media) were capable of reducing Benedict's solution at 100°C. With very few exceptions the reactions were strong and were readily obtained after two or three days' incubation at 27°C. Table 15 outlines the results obtained. These are analysed according to source of the organisms as in the previous experiment.

Results

Of particular note are the figures quoted for the strains isolated from sources other than plants (C) and for authentic pathogens (D). Of the former the proportion giving reduction was small, of the latter large. A large proportion of the general plant isolates (B) also gave a positive result. A correlation between acid production and copper reduction seemed possible but on detailed examination the two characters appeared to be independent as a reducing reaction was obtained in some cases without acid formation and vice versa. The majority of the pseudomonads tested were, however, either positive or negative for both characters (Table 16). The mors-prunorum and syringae strains all gave a reducing reaction with the exception of the two of the latter species which did not utilise sucrose.

Experiment 16/

TABLE 15

Copper reduction tests.

(1% sucrose in inorganic, peptone, or tryptone medium)

A.	All sources.		%
	Organisms tested	204	100
	Organisms reducing	96	47
B.	Isolations from plants.		
	Organisms tested	158	100
	Organisms reducing	91	57
C.	Isolations from sources other than plants.		
	Organisms tested	46	100
	Organisms reducing	5	11
D.	Authentic pathogens.		
	Organisms tested	35	100
	Organisms reducing	33	92

TABLE 16

The relationship between acid production and copper reduction in sucrose-peptone and sucrose-tryptone media.

Number of organisms tested ... 204

Medium	Reactions							
	A +	R +	A +	R -	A -	R +	A -	R -
Sucrose- - peptone	61		13		35		95	
Sucrose- -tryptone	97		23		9		75	

Key A ... acid (B.C.P.)

R ... reduction of Benedict's solution at 100'C.

Experiment 16 The production of levan.

Introduction

The ability of some pseudomonads to synthesise polysaccharide has been known for a considerable time. This character is readily recognised by the production of large mucoid colonies on sucrose agar.

Wormald (1932) used a nutrient agar containing 5% sucrose for the isolation of Ps. prunicola. The polysaccharide-forming ability of that organism distinguished it from accompanying saprophytes. Cooper & Preston (1935) and Crosse (1953) found that many bacteria pathogenic to plants synthesise, from sucorse, polysaccharides which are of the fructosan type and analogous to the levans formed by Bacillus mesentericus and B. subtilis. Polysaccharide formation was examined by Erikson (1945) with reference to Ps. mors-prunorum, and she noted that Ps. fluorescens, Ps. pyocyaneus, Ps. cerasi and Ps. marginalis did not produce raised gummy growths in the presence of sucrose. Crosse (1953) also used the character as an aid to the identification of Ps. mors-prunorum. Apart from Erikson, no other investigator appears to have examined pseudomonads other than the plant pathogens for this property. Consequently, its significance as a differential character for the few species in which it has been detected is not necessarily secure. The strains under study were therefore examined for their ability to produce levan.

A/

A series (201) of strains were surface-plated on nutrient agar containing 5% sucrose so as to provide discrete colonies after 2 days incubation at 27°C. The colonies of each strain were examined and those which showed the mucoid colony appearance were noted.

Results

In the case of a few strains, the polysaccharide was not so abundant as normal and a decision was difficult. If thepolysaccharide formation was active the colony was at first a glistening hemisphere. There followed, on continued incubation, a softening and then a liquefying of the polysaccharide. The colony then lost all rigidity, spread over the agar and dropped on the cover of the Petri-dish. This apparent de-polymerisation of the polysaccharide occurred at varying periods of from 24 hrs. to 10 days according to strain. The colonies which only remained turgid for a short time were called "watery" colonies. Table 17 shows the distribution of various types of colony on sucrose agar. The majority of the colonies forming levan were isolated from plant sources and a particularly high percentage of these was found among the pathogens. None of the pathogens produced the "watery"-type colony. The strains of Ps. mors-prunorum all produced levan colonies, but, of the eleven syringae strains there were three exceptions. Two of these were unlikely to produce levan/

TABLE 17

The production of levan-containing colonies.

(medium - 5% sucrose in nutrient agar)

A.	All sources.		%
	Organisms tested	201	100
	Organisms producing levan colonies	117	58
B.	From plants.		
	Organisms tested	155	100
	Organisms producing levan colonies	106	68
	(including "watery-type" colonies - 25)		
C.	From sources other than plants		
	Organisms tested	46	100
	Organisms producing levan colonies	11	24
	(including "watery-type" colonies - 5)		
D.	Authentic pathogens.		
	Organisms tested	36	100
	Organisms producing levan colonies	31	86
	(no "watery-type" colonies formed)		

TABLE 18

The relationship between levan formation and copper reduction in sucrose media

(Number of organisms tested, 201)

Levan formation	Reduction	Number of organisms
+	+	91
+	-	26
-	+	8
-	-	76

Levan formation was determined visually by the appearance of colonies on nutrient agar containing 5% sucrose.

The reducing reaction was determined by means of the Benedict's qualitative reagent on cultures in a solution containing 1% sucrose and 0.5% peptone.

The tests were carried out in both cases after an incubation of 48 hr. at 27°C.

levan from sucrose as they did not utilise sucrose as a sole source of carbon. The remaining strain, however, produced acid from sucrose.

Experiment 17 Levan-reduction relationship.

The relationship between levan-formation and the copper reducing properties of the cultures in the medium was considered, (Table 18). Although the majority of the strains are either positive or negative for both properties a small number show a positive for one and a negative for the other. One of these is the syringae strain mentioned in the previous experiment which, although utilising sucrose, did not produce levan.

Experiment 18

A comparison of the reducing properties of cultures in gluconate and sucrose media.

Introduction

Lockwood, Tabenkin & Ward (1941) reported the production of 2-ketogluconate from glucose by several strains of Pseudomonas. Norris & Campbell (1949) identified 2-ketogluconate as an intermediate in the oxidation of glucose. According to Koepsell (1950) the oxidation of gluconate characteristically begins with the rapid formation of 2-ketogluconate. When the ketogluconate is oxidised, carbon/

carbon dioxide, a-ketoglutarate, and pyruvate appear. Haynes (1951) suggested that the reducing compound that appears during the oxidation of potassium gluconate in shaken culture is 2-ketogluconate. This presumption was probably correct as Koepsell was unable to detect any other copper-reducing substance during the process of gluconate fermentation.

Entner & Stanier (1951) gave reasons for suggesting that the formation of the keto-acid is not on the main pathway of gluconate metabolism and later Wood & Schwerdt (1953) observed that cell-free extracts of glucose-grown Ps. fluorescens contained the enzymes necessary for the oxidation of glucose, glucose-1-phosphate, 6-phosphogluconate, ribose-5-phosphate and gluconate and suggested that the production of 2-ketogluconate was the result of a side reaction. The intact cells of the pseudomonads oxidised 2-ketogluconate slowly when it was used as a sole carbon source (Campbell, 1954). The oxidation of glucose to the 2-keto-acid did not appear to involve a phosphorylation (Stokes & Campbell, 1951; and, Sebek & Randles, 1952). The keto-acid may be subsequently phosphorylated and then degraded to pyruvate (Gunsalus, Horecker & Wood, 1955).

Katznelson (1955) in a study of the metabolism of some phytopathogenic bacteria observed that, although gluconate was utilised by all the species tested except those of the genus Xanthomonas, 2-ketogluconate was less widely used and by/

by none of the pseudomonads examined by him, all of which were phytopathogens. A development of the work carried out by Haynes (1951) has been described by Cowan (1955) whereby a microtest involving the optimum conditions for gluconate oxidation was introduced.

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When a selection of the organisms used in the present work were grown on the gluconate medium suggested by Haynes and the cultures tested with Benedict's solution for reducing substances an unexpected result was obtained. A positive test from gluconate appeared to bear little or no relationship to one from sucrose. An indication of the results obtained is given in Table 19. Only three of the eleven strains tested were positive in both media. These three organisms consisted of Ps. marginalis (P_3) and two of the saprophytic strains. The cultures which produced a copper-reducing action from sucrose and not from gluconate were all pathogens, including both mors-prunorum and syringae. The opposite results were shown by the remaining four strains which, it was noted, gave consistently negative results with sucrose (see Tables 10, 11 and 12).

Discussion

The reducing substance obtained from gluconate was considered by Koepsell (1950) to be 2-ketogluconate. It might/

TABLE 19

The production of reducing substances from gluconate and from sucrose

Organism	Gluconate	Sucrose
P1	-	X
2	-	X
3	X	X
4	-	X
5	X	-
6	-	X
8	X	X
9	X	X
10	X	-
11	X	-
12	X	-

might be supposed that when sucrose is utilised by the same organisms the fructose is converted to levan (non-reducing) and the glucose is metabolised through gluconate. This should provide at least some ketogluconate and give a reducing reaction. However, it can be seen from Table 19 that a reducing reaction is obtained from sucrose but not from gluconate in a number of cases. This suggests that an additional reducing substance may be involved. The only probable one is fructose which, could be released from the levan if sufficient depolymerisation occurs or, alternatively, may not be entirely converted into levan at some stage of the metabolic process.

Experiment 19 The utilisation of 2-ketogluconate.

The eleven representative strains used in previous experiments were inoculated into an ammonium salt medium (Medium (B) of Experiment 14) containing 0.1% calcium 2-ketogluconate.

Daily tests with Benedict's solution were carried out on portions of the cultures during an incubation of 6 days at 27°C.

Results

These revealed that the substance was utilised by those organisms that produce a reducing substance from gluconate (presumably the same keto-acid) (Table 20). The group of organisms/

TABLE 20

The utilisation of 2-ketogluconate (0.1%)

in an ammonium salts medium

Organism	Copper-Reduction Test		
	1	2	3 - 6 days
P1	X	X	X
2	X	X	X
3	X/-	-	-
4	X	X	X
5	X	X/-	-
6	X	X	X
8	X	X/-	-
9	X	X/-	-
10	X/-	-	-
11	X/-	-	-
12	X/-	-	-
control	X	X	X

organisms which did not utilise the ketogluconate was the same as that which produced a negative result from gluconate and a positive from sucrose in the last experiment (Table 19), i.e., a group made up entirely of pathogens, including the mors-prunorum and syringae strains. Exactly similar results were obtained using suspensions of the organisms in a phosphate buffer (pH 6.8) containing M/100 calcium 2-keto-gluconate.

Discussion

Although the four organisms which failed to utilise the keto-acid are all pathogens it appears that this inability is not a reliable indication of pathogenic properties since one of the other seven which did utilise the acid (P_5) was Ps. viridiflava. A further culture of this species was obtained from the same source (Dowson (Cambridge)), and was found to give an identical result. The fact that Katznelson (1955) found that none of the pathogenic pseudomonads he tested were capable of utilising 2-ketogluconate could therefore be misleading. He did however suggest that the enzymic system necessary for its oxidation was present in the cells and that the inability of the intact cells to utilise it was due to permeability barriers.

Experiment 20/

Experiment 20 Chromatographic Studies.

Introduction

It had become increasingly obvious that an identification of the reducing substance(s) produced from sucrose should be made. For this purpose a modification of Proom & Woiwood's (1951) method of Petri dish chromatography was found to be suitable.

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The apparatus used consisted of an inverted large glass lid (diameter 14 cm.) containing a Petri dish lid (diameter 10 cm.). The outer container was covered by a sheet of plain glass. The solvent mixture was contained in the Petri dish lid and water in the outer lid. The method consisted of "spotting-on" a drop of undiluted culture fluid near the apex of each of the four segments of a Whatman No. 1 filter paper (diameter 11 cm.). The paper was allowed to dry at room temperature; and a wick of rolled filter paper inserted through a hole in the exact centre. The paper was then placed centrally on the Petri dish lid with the wick in contact with the solvent. The apparatus was immediately covered. (Figure 4a).

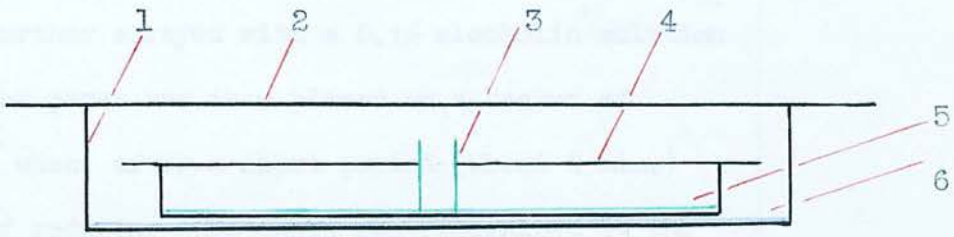
The solvent consisted of n-butanol, pyridine and water (3:1:1). The chromatogram was run for 3 hrs. at room temperature when the paper was removed and dried rapidly at/

Figure 4

The Petri-dish chromatographic method adopted in the studies of carbohydrate dissimilation.

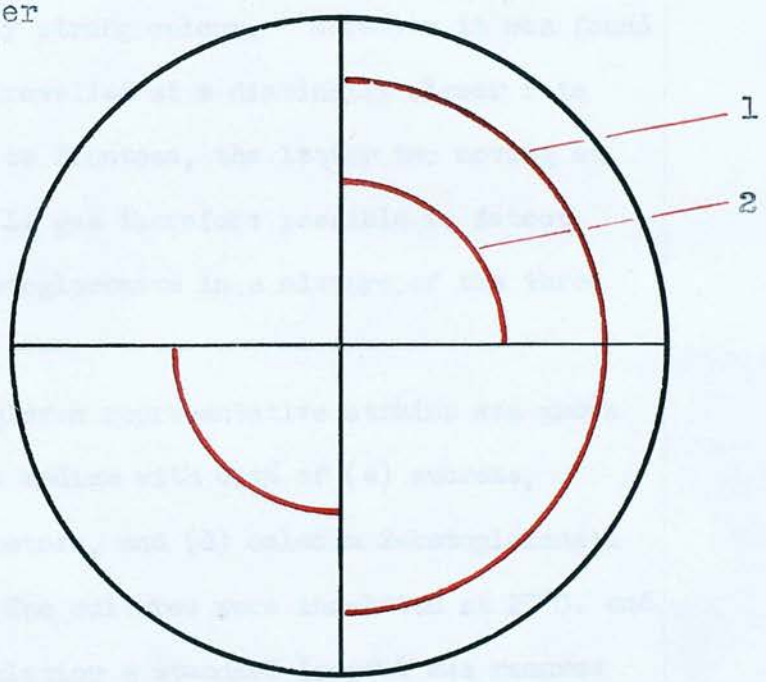
(based on, Proom & Woiwood (1951))

a) Apparatus.



Key: 1 Petri-lid
2 Glass plate
3 Wick (filter-paper)
4 Filter-paper
5 Solvent
6 Water

b) Appearance of paper after treatment



The reactions obtained from sucrose cultures are depicted as;

1 The reaction given by hexoses.
2 The reaction given by 2-keto-gluconate.

at 45°C in a fume cupboard. The paper was sprayed first with a 0.5% chloroform solution of triphenyltetrazolium chloride (Trevelyan, Procter & Harrison, 1950), allowed to dry and then further sprayed with a 0.1% alcoholic solution hydroxide. The paper was then placed on a beaker of boiling water, when, after a short period (about 2 min.) the position of reducing substances was recognised by the appearance of purplish-red bands (Figure 4b.). A very slight background stain generally developed but caused no confusion.

Preliminary tests showed that glucose, fructose, and 2-ketogluconate reacted excellently to the reagent, fructose giving a particularly strong colour. Moreover it was found that the keto-acid travelled at a distinctly slower rate than either glucose or fructose, the latter two moving at identical speeds. It was therefore possible to detect the presence of 2-ketogluconate in a mixture of the three substances.

The series of eleven representative strains was grown in the ammonium salt medium with 0.1% of (a) sucrose, (b) glucose, (c) fructose, and (d) calcium 2-ketogluconate as carbon source. The cultures were incubated at 27°C. and each day after inoculation a standard loopful was removed from each and spotted on to a filter paper in the manner described above. The resultant chromatograms revealed significant differences among the strains tested, (Table 21).

Results/

TABLE 21

The results of the chromatographic analysis of products of the dissimilation of sugars, etc.

A. SUCROSE

Organisms	Days	Hexose	2-keto--gluconate
P 1, 2, 4, & 6.	1-16	XX	-
P 3.	1	X	-
	2	XX	-
	3	XX	X
	4	XX	X
	11	XX	-
	16	XX	-
P 5, 10, 11 & 12	1-16	-	-
P 8 & 9.	1	X	-
	2	XX	X
	3	XX	XX
	4	XX	XX
	11	XX	XX
	16	XX	X

B. GLUCOSE

Organisms	Days	Hexose	2-keto--gluconate
P 1,2,4, 5 & 6	1	XX	-
	2	XX	-
	3	X	-
	4	X	-
	8	-	-
P 3.	1	XX	XX
	2	X	XX
	3	-	XX
	4	-	-
	8	-	-
P 8.	1-8	XX	-

TABLE 21 (continued)

B. GLUCOSE (continued)

Organisms	Days	Hexose	2-keto--gluconate
P 8 (+ fructose)	1	XX	-
	2	XX	-
	3	XX	X
	4	XX	XX
	8	X	XX
P 9,10,11 & 12	1	XX	XX
	2	X	XX
	3	X	X
	4	-	X
	8	-	-

C. FRUCTOSE

Organisms	Days	Hexose
P 1,2,4,6,11 & 12.	1-8	XX
P 3,5,9, & 10.	1	XX
	2	XX
	3	XX
	4	XX
	8	X
P 8.	1	XX
	2	XX
	3	XX
	4	XX
	8	-

D. 2-KETOGLUCONATE

Organisms	Days	2-KG
P 1, 2, 4 & 6	1-8	XX
P 3,5,8,9,10,11 & 12	1-8	-

TABLE 21 (continued)

E. GLUCONATE

Organisms	Days	2-KG
P 1, 2, 4 & 6	1-4	-
P 3, 5, 8,	1	X
9, 10, 11, & 12	2-4	XX

Key

XX ... strong reaction

X ... weak reaction

- ... no reaction

Results

Calcium 2-ketogluconate: The strains were divided sharply into two groups by reason of their utilisation or non-utilisation of the compound. The division was identical to that obtained by the culture method described in Experiment 19.

Sucrose: Two different groupings were revealed, again on the basis of utilisation. Of those which had the ability to use sucrose two sub-groups were recognised, viz. those which produced 2-ketogluconate and those which did not. The latter sub-group identified itself with the strains which did not utilise the keto-acid.

Glucose: This sugar was utilised readily by all the strains except P_8 which was observed to grow only slowly in the glucose medium. Those strains which produced 2-ketogluconate were, with the exception of P_3, non-pathogens and included some of those which did not utilise sucrose.

Fructose: The results of the four-day examination shown in Table 21 revealed a distinct lack of activity towards fructose. Later examinations of the cultures by the same chromatographic procedure, however, showed that the sugar was slowly utilised by some of the strains after approximately 8 days without the production of further reducing substances.

Discussion

The main outcome of this experiment was the identification of the reducing substances formed by the organisms from sucrose/

sucrose. Free hexose(s) was found to be present with or without the keto-acid. This would explain the differences obtained on comparing the reducing properties of gluconate and sucrose cultures (Table 19). It appears, therefore, that among the pseudomonads only a proportion have the ability to produce 2-ketogluconate in the breakdown of glucose.

Two complicating features arose from the results. The use of sucrose as a substrate for the production and subsequent detection of the keto-acid is obviously only valid when the particular organism under test is known to utilise that sugar. The second feature is interesting in that it appears to involve some form of mutation. It was noted in the preliminary experiments that strain P_8 was capable of dissimilating glucose with ease (Table 11). During this last experiment, however, the same organism preferred sucrose or fructose to glucose for growth. As can be seen from Table 21 (B) no keto-acid was produced from glucose alone but only when fructose was added. (The organism was capable of using fructose slowly as a sole source of energy without forming 2-ketogluconate - see Table 21 (C).) The keto-acid was also produced by the same organism from gluconate, a substrate which supported good growth. It will therefore be necessary if 2-keto-acid production is used as a differential character among these bacteria that due/

due care be taken in the choice of the substrate. Sucrose and glucose are, apparently, not dependable. Gluconate, on the other hand, appears to be a reliable substrate. In view of the results obtained using 2-ketogluconate as the carbon source it is possible that all the organisms which can produce that acid can also break it down. As only a small number of strains were used for the experiment such a suggestion must be regarded as tentative.

Experiment 21.

The absence of 2-ketogluconate in sucrose cultures of Ps. mors-prunorum and Ps. syringae.

It was observed in the previous experiment that a strain of Ps. mors-prunorum (P_6) and one of Ps. syringae (P_1) could, in addition to other pathogens utilise sucrose without producing 2-ketogluconate. This was further tested with all the available strains of the two species.

An ammonium salt medium containing 0.1% sucrose was inoculated with 6 strains of Ps. mors-prunorum and the 9 strains of Ps. syringae which could utilise sucrose as a sole source of carbon. After an incubation of 4 days at 27°C. chromatograms were prepared as in the previous experiment.

Results

The results showed that all the strains tested did not produce 2-ketogluconate.

Conclusions/

Conclusions (Experiments 13 - 21)

With glucose, lactose and sucrose some differentiation on the basis of acid production would be possible only if rigidly defined media were used under particular conditions of temperature and time of incubation.

Several properties of the strains examined appeared to be worthy of note, viz.,

(a) the utilisation of sucrose as the sole carbon source in an ammonium salt medium with or without the production of acid.

(b) the formation of 2-ketogluconate from gluconate (as detected by a copper reduction test at 100°C).

(c) the formation of levan (as detected on 5% sucrose agar).

The mere detection of acid and copper-reducing substances in sucrose cultures are not considered to be sufficiently well defined to be regarded as important differential characters. The detection of the latter is only of value if the substance is actually identified as, for example, in Experiment 21.

PART III

The lipolytic activity of the organisms.

The lipolytic activity of the organisms.

Introduction

The ability of some members of the genus Pseudomonas to hydrolyse fats is well known and accounts for many cases of spoilage in commercial products of a fatty nature such as butter.

Starr & Burkholder (1942) made a study of the lipolytic activity of the phytopathogenic bacteria, including Pseudomonas, using the spirit blue agar method proposed by Starr (1941). Of 27 species tested only 4 were found to be lipolytic. These authors cast some doubt on the validity of the latter as true phytopathogenic Pseudomonas, one being closely related to a typical saprophyte and the others being classified unsatisfactorily. The saprophytic species were said to be mostly lipolytic. In the above paper and in subsequent publications (e.g. Burkholder & Starr, 1948) the authors made it clear that the term "lipolytic" was used by them in a limited sense, being based on changes in the medium used. They point out that the cottonseed oil substrate used in the medium could possibly be decomposed in ways which would not give the characteristic colour change of the indicator.

Starr's method involved the incorporation of aniline blue as an indicator in a cottonseed oil emulsion agar. The medium was stained a lavender colour while lipolytic colonies/

colonies produced zones of a deep blue colour.

Experiment 22. A survey of previous methods.

It was considered expedient to compare several of the techniques suggested for the detection of lipolysis. A selection of lipolytic and non-lipolytic representatives of various bacterial genera and some moulds were used in this preliminary work. Cottonseed oil was used as an emulsion in an ordinary nutrient agar in the first experiments. The methods were as follows:-

(a) The copper sulphate method. (Berry, 1933).

Colonies of the organisms grown on plates of emulsion agar were flooded with a saturated solution of copper sulphate for 10 min. and rinsed gently with water. In this method the intensity of the colour produced around the lipolytic colonies is regarded as a gauge of the lipolytic activity of the organisms.

(b) The use of solutions of dyes as washes. (Long & Hammer, 1937).

This method was introduced as a means of using dyes to detect changes in fat media while avoiding any inhibitory effects associated with the incorporation of dyes in the media. Solutions, at appropriate concentrations, of various dyes, both basic and acidic, were flooded on to the plate cultures for periods up to 1 hr. The dye solutions were then washed off with tap-water and the changes were noted

by/

by naked eye and under low power.

(c) The use of dyes incorporated into the fat medium.

(Turner, 1927, and Long & Hammer, 1937)

This method was tested although it is known to be unsatisfactory if the growth of an organism is inhibited by the dye. Several dyes, as in (b), were used. Firstly, the concentration of each dye which gave a satisfactory colour to the fat medium was found. This was achieved by incorporating 1 ml. of decimal dilutions of the dyes into melted agar. After mixing, plates were poured and examined, and the concentration giving a suitable colour was noted for each dye. Batches of emulsion agar containing the dyes were prepared, inoculated, incubated for 3 days at 30°C., and the reactions examined.

Summary of results and conclusions.

(a) The copper sulphate method.

In general the results were weak, and although lipolytic and non-lipolytic growths could be differentiated, there seemed room for marked improvement. The copper soap formed in, around and under the lipolytic colonies but in no case was an entire halo or border formed. In the parts of the medium around the perimeter of the growths which showed no copper soap distinct indications of lipolysis could be detected by low power examination. The fact that

no/

no reaction with copper had taken place in these areas remained unexplained and attempts to control the variations were unsuccessful.

(b) The use of solutions of dyes as washes.

The basic dyes used showed a varied affinity for the globules around lipolytic growths. Bacterial colonies and the agar medium was also variably stained, but the effect was not such as to interfere greatly with the detection of lipolytic activity. Normally the agar tended to be less deeply stained in a zone around all the growths, both lipolytic and non-lipolytic. The acid dyes gave a general colour to the whole plate with no differentiation. As the basis of a method of detection none of the dye reactions was considered sufficiently sensitive.

(c) The use of dyes incorporated into the media.

As in (b) the basic, but not the acidic, dyes were found to stain the products of lipolysis. The reading of the results did, however, require experience, and a low power examination was necessary. Several of the dyes faded to an unsatisfactory degree in the fat emulsion agar, and several of the more promising ones were found to be inhibitory.

- - - -

The/

The concentration and degree of emulsification of the fat appeared to be important factors in the detection methods. For spot inoculations which give rise to large colonies a fat or oil concentration of 2% was suitable, and the clearest results were obtained when fat globules were of a size still visible to the naked eye. On the other hand, in cases where the inoculum was spread to give small discrete colonies, a finer emulsion and a higher fat concentration were desirable so as to allow even the smallest colonies to contact a number of fat globules.

Experiment 23 The use of hydrogenated fats.

In the last experiment it was noted that considerable fading took place during the incubation period with the dye fat media. This was particularly true of the two dyes found to have most promise, viz., Victoria blue and night blue. This phenomenon was found to take place most rapidly when natural fats or oils containing unsaturated components were used in the media, e.g. cottonseed oil, butter fat, castor oil, or olive oil. This suggested that peroxides may possibly have formed and have reacted with the dyes to form colourless compounds.

By using, in place of the highly unsaturated oils, a hydrogenated natural fat such as is obtained from margarine, the stability of the medium was greatly increased. This work/

work was described in a paper presented before a meeting of the Society for Applied Bacteriologists and a summary of it was subsequently published, (Paton & Gibson, 1953).

Experiment 24 The use of Victoria blue base.

An improvement in the colour contrast obtained in dye fat media was brought about by adopting the use of the base of Victoria blue as the indicator (Richards, 1946 and Jones & Richards, 1952).

Victoria blue, which is soluble in water, forms, in the presence of alkali, a base which is insoluble in water yet soluble in fats, oils and their solvents. In contrast to the blue water-soluble dye the base, in solution, is red in colour. The inhibitory action of the dye, although less than that of most of the others tested, was weakened still further by dissolving the base in the fat. On the lipolytic breakdown of the fat the red dye reverts to the water-soluble blue form which stains fatty acids and gives a very satisfactory colour contrast.

Experiment 25 The preparation of the fat medium.

As a result of the information obtained from the previous experiments an effort was made to prepare a medium which would be reasonably stable and would give the adequate colour contrasts. The following is a brief description of the/

the procedure finally adopted:-

Good quality margarine (approx. 100 g.) is melted in a flask containing 200 ml. tap water and a few glass beads. The mixture is boiled for 5 min. and 0.2 g. of powdered Victoria blue* is added. Gentle boiling is continued for 20 min. during which time the fat will become red and the water blue. A few drops of 10% sodium hydroxide are added in order to convert the remaining water-soluble blue dye into the fat-soluble and red dye base. The boiling is continued for a further 10 min. when the fat will have become saturated with the dye base and will have acquired a deep red colour. The contents of the flask are then passed through coarse filter paper at about 50°C. This process is slow and it is convenient to carry out the filtration overnight in an incubator. The dye fat is decanted and may be sterilised at 22½ lb. pressure when it should remain stable for some months particularly if stored in cool, dark conditions.

To prepare emulsion agar plates it is necessary to use a nutrient agar with a final pH not lower than 7.2. Sufficient dye fat is added to screw-capped bottles of the nutrient agar to give a fat concentration of about 5%. After sterilisation the medium is cooled to 45°C., shaken to produce an emulsion of the fat in the agar, and poured as/

*Victoria blue; The British Drug Houses, Ltd., London.

as thin plates. The quality of the completed plate depends largely on the size of the fat globules. If the globules are too large or the medium too hot on pouring an excess of fat rises to the surface before the agar sets.

Experiment 26 A rapid test for lipolysis.

A rapid spot test was evolved whereby the lipolytic activity of an organism could be detected in a much shorter time than that required by a culture method.

The growth from an ordinary nutrient agar slope culture is converted to a thin paste with sterile water. This is mixed roughly into a solidified drop of the dye fat (prepared as in the previous experiment) on the under surface of the lid of a Petri-dish. The base of the dish should contain a moist filter paper. After an incubation of 3 hrs. at 27° or 30°C. an actively lipolytic organism will produce a distinct blue colour in the mixture. Weaker strains will show the reaction within 8 - 10 hrs.

Experiment 27 The lipolytic activity of Pseudomonas.

Having obtained a convenient and reliable method for detecting lipolysis, strains of Pseudomonas were tested on the Victoria blue base margarine-fat agar. The results were recorded after incubation for 48 hrs. and are summarised in Table 22.

Results/

TABLE 22

The lipolytic activity of a series of strains as judged by means of Victoria blue base and hydrogenated fat

			%
A.	All sources.		
	Organisms tested	204	100
	Organisms showing lipolysis	89	44
B.	Isolation from plants.		
	Organisms tested	158	100
	Organisms showing lipolysis	63	40
C.	Isolations from sources other than plants.		
	Organisms tested	46	100
	Organisms showing lipolysis	24	52
D.	Authentic pathogens.		
	Organisms tested	35	100
	Organisms showing lipolysis	2	5

Results

The reactions observed on the medium were clear and definite. The ability to hydrolyse fat did not appear to be related to the sources of the organisms. It was noted, however, that only a very small percentage of the authentic pathogens showed a positive reaction. The two pathogenic strains which proved to be lipolytic are Ps. marginalis (P_3) and one of Ps. syringae. The first-mentioned organism, it may be noted, is commonly regarded as a facultative pathogen more closely related to the saprophytes in many ways and which only produces disease under abnormal environmental conditions. The lipolytic activity of a strain of Ps. syringae was, however, unexpected. Starr & Burkholder (1942) found that none of the 11 isolates of Ps. syringae which they tested were lipolytic. An explanation of this discrepancy has not been obtained.

PART IV

Miscellaneous Properties of the organisms.

Miscellaneous Properties of the Organisms

Experiment 28 Denitrification

Seleen & Stark (1943) found a uniformity of the reduction of nitrates to nitrogen or gaseous nitrogen compounds and pointed out discrepancies in the reduction to nitrites.

The ability of a number of the organisms in this study to reduce nitrates to gaseous products was found to give clear results which showed no variation after several years of frequent sub-culturing.

Of the 204 strains tested 18 (9%) produced gas from nitrate peptone water at 27°C. None of the pathogens in the series showed gas formation from nitrate.

Experiment 29 Action on milk.

Introduction

Clara (1934) suggested that the variable reports on the action of pseudomonads on milk was probably due to differences in the interpretation of "curdling" and "peptonisation". He reported that all the cultures tested turned milk alkaline to brom cresol purple but produced no curd or peptonisation. The only variations noted were in the rate of change. This appears to be an over-simplification of the position of the organisms. Seleen & Stark (1943) divided members of the genus into three groups according

to/

to their action on litmus milk, viz., (a) digestion of the casein, (b) no digestion but a basic reaction, and (c) no obvious reaction. The actions of the pseudomonads on litmus milk were considered by Gaby (1955) to be very variable.

An examination of the reactions of the strains on litmus milk revealed a grouping somewhat similar to that described by Seleen & Stark, viz.,

(i) The formation of rennet-like curd which subsequently contracted prior to digestion.

(ii) The clearing of the medium without the prior formation of a curd.

(iii) No obvious action on the milk other than a slight alkalinity.

A number of group (iii) reduced the litmus to a colourless compound. These cultures were shown to be alkaline by using brom cresol purple instead of litmus.

None of the reactions appeared to be associated with the organisms derived from any particular source. All the strains of Ps. mors-prunorum and all but one of Ps. syringae produced a clearing of the medium as in group (ii) above. The exception produced a curd which was subsequently digested.

The results obtained did not vary from those obtained in similar experiments carried out over a period of three years.

TABLE 23/

TABLE 23

The action of pseudomonads on litmus milk.

Reaction	No.	%
K P_c	83	46
R P_c	6	3
K P_s	30	17
R P_s	2	1
K P_o	38	21
R P_o	3	2
N C	18	10

KEY:-

P_c curd and subsequent digestion.

P_s clearing.

P_o no evidence of digestion.

NC no change observed.

R reduction of litmus.

K alkaline.

Total number of cultures tested, 180.

Experiment 30 Action on egg-yolk.

Introduction

The lecithinase activity of bacteria has been studied with reference to the genus Bacillus (McGaughey & Chu, 1948). The presence of a similar, if not identical, enzyme system among members of the genus Pseudomonas has been investigated by Villecourt & Jacobelli (1953). The positive reactions obtained were comparable in appearance with that obtained with Bacillus cereus. In some cases the organisms (all proteolytic) produced a clearing of the medium with or without the lecithinase reaction. It was emphasised that the/

the reaction, when it occurred, was a stable one.

A series of 200 strains was tested on the egg-yolk medium described by McGaughey & Chu (1948). Only definite opaque zones bordering on the growths were accepted as evidence of lecithinase activity. Clearing of the medium around the colonies was also noted. The results are summarised in Table 24.

Results

These show that the positive results are fairly evenly distributed between the general plant isolates (which include the pathogens) and the isolates from other sources (21% and 18% respectively). It is interesting to note that out of 35 authentic pathogens none produced a lecithinase reaction. Clearing of the medium was not always associated with proteolytic or lipolytic activity in this series of organisms.

Experiment 31 Haemolytic activity.

Introduction

According to Haynes (1951) the suggestion that Ps. aeruginosa has the characteristic ability to haemolyse the blood cells of certain warm-blooded animals is of limited usefulness in the absence of information to show that other pseudomonads which grow at 37°C fail to haemolyse blood. The majority of the Pseudomonas studied by Villecourt & Jacobelli (1953) produced a distinct haemolysis of sheep cells/

TABLE 24

The lecithinase reaction of 200 strains

	Origin of isolates			
	a. General plant isolates	b. Authentic plant pathogens	c. Isolates from other sources	a+c Total
L	7	0	5	12
LC	26	0	3	29
C	37	2	17	54
N	86	33	19	105

Key

L ... positive lecithinase reaction.

LC... positive lecithinase reaction and clearing.

C ... clearing only.

N ... no visible reaction.

cells and in some cases the appearance obtained was identical with that given by Ps. aeruginosa. None of the strains used, apart from Ps. aeruginosa, grew at 42°C (a specific character of that organism).

The usual series of strains were plated on a medium made up of nutrient agar with the addition of 0.5% sodium chloride and 5% bovine blood. The plates were incubated up to 4 days at 27°C. and examined for haemolysis.

Results

Only 20, all non-pathogens, of the 200 cultures tested showed some degree of haemolysis and of these 5 produced a wide zone of activity around the colonies. None of the strains which showed a positive result was also capable of growing at 42°C., despite the fact that they all grew slowly at 37°, thus showing that Ps. aeruginosa was not represented in the series.

Only one of the 200 strains used in the above experiment is capable of growing at 42°C. This is an apyocyanogenic strain of Ps. aeruginosa. - It has no action on bovine blood.

Experiment 32 Antibiotics as aids to identification.

Introduction

A scheme for the differentiation of the Pseudomonadaceae (Bergey) resulted from work with marine bacteria by Shewan, Hodgkiss/

Hodgkiss & Liston (1954). These workers suggested that differences in sensitivity to penicillin and terramycin are taxonomically useful properties.

The method was investigated using a series of 200 strains. Solutions of penicillin (75 i.u./ml.) and terramycin (300 mcg./ml.) were prepared, sterilised by filtration, and dispensed in 0.1 ml. quantities in cups cut in nutrient agar plates, previously seeded with 24 hr. broth cultures of the organisms.

Observations of zones of inhibition were made after a 24 hr. incubation at 27°C.

Results

Table 25 shows that the four possible combinations of results were obtained. There was no evidence of a relationship between any of the groups so formed, and pigment formation or other characteristics of the organisms studied.

TABLE 25

The sensitivity of 200 strains to penicillin and terramycin.

Penicillin (75 i.u./ml.)	Terramycin (300 mcg./ml.)	No. sensitive	%
-	-	23	12
-	x	137	68
x	x	37	18
x	-	3	2

PART V

Colony variation.

Colony Variation.

Introduction

Wormald (1932) regarded the colony appearance on 5% sucrose agar as an important test in identifying Ps. mors-prunorum. The stability of the appearance of a colony must be considered as important in assessing the value of this test.

The literature contains several reports on variation in Pseudomonas. Wilson (1934) separated from Ps. cerasi a colony which was pathogenic but not as markedly so as the "parent" strain. The cultural features of the variant, as far as they were studied were virtually the same as those of the "parent" strain apart from minor differences such as the amount of acid produced from sucrose and glycerin. Ark (1946) was able to induce a mutation in certain phytopathogenic bacteria by means of acenaphthene, but failed to demonstrate the change in the only Pseudomonas tested. Chodat & Wassilieff (1950,1951) described a dissociation of Ps. fluorescens into S and R forms. This phenomenon was independent of the nature of the culture medium which did, however, condition the expression of the R form. On plating on peptone agar two kinds of colony appeared, viz., the smooth (S) and wrinkled (R) forms.

Smooth (S), mucoid (M), and filamentous (F) colonial varients were isolated by Williamson (1955) from a strain of Ps. aeruginosa. The S and F mutants were unencapsulated and/

and fermented arabinose and galactose while the remaining mutant (M) possessed capsules and did not ferment these sugars. On the basis of equal numbers of cells the mucoid strain was approximately twelve times as virulent for mice as the filamentous strain.

Experiment 33 The incidence of colony variation.

The stability of colony structure was examined in a series of strains. Each was plated on ordinary nutrient agar with and without 5% sucrose from 9 month-old stock cultures. After 48 hrs. at 27°C. the colonies were examined by naked eye and low power.

Results

Of 204 cultures tested in this way 41 showed at least two types of colony and a few showed three types. Although the variants were usually obvious on both media they were observed more frequently and more easily on the sucrose medium. The variations most commonly noted were changes from semi-opaque to dense, or from entire in outline to diffuse. On sucrose agar a variation in the structure of the colonies in which levan was formed could be recognised under low power. In this case the changes concerned the type of flecking on the convolutions and radial striations.

The appearance of colony variants was not observed in cultures from any particular source. Among the mors-

prunorum/

prunorum and syringae strains at least one of each showed differences in colony structure. One of these was selected for further study.

Experiment 34

Colony variants of a strain of Ps. mors-prunorum.

The strain selected for this experiment was P6 (Ps. mors-prunorum).

On plating on nutrient agar two types of colony appeared which were examined after 2 days' incubation at 27°C.

Type A: Normal appearance; amorphous, semi-opaque, raised, greyish-white colony. This type made up most of the growth.

Type B: Amorphous, opaque, greyish, raised colony. Not easily emulsifiable. Only 3 or 4 colonies of this type appeared on each plate.

Nutrient broth was inoculated from the original culture, incubated for 3 days and plated on nutrient agar. Again, types A and B appeared, the latter in greater numbers than before, but, in addition, a further type (C) was revealed in very small numbers.

Type C: Flat, irregularly-shaped colony which appeared, under low power as bundles of short wavy threads. The colony was similar in colour and opacity to type A.

On/

On isolating the 3 types and replating on nutrient agar variants again appeared as follows:

Types plated	Resultant colonies
A	A, B and, occasionally, C.
B	A and B.
C	C.

Despite frequent attempts to obtain a reversion type C remained uniform.

On sucrose agar the types were recognised by the marked differences in the shape of the levan colonies, especially when incubated for about 7 days. Type A produced hemispherical colonies while B produced peaked and markedly raised dome-like colonies. C was conspicuously flatter but still obviously a levan producer.

A microscopic examination showed that A was made up of short, actively motile rods; B of longer rods, motile by means of a slow twisting motion; and type C mainly of long filaments which were again very slowly motile.

A comparison of some of the biochemical activities of the three types was made. Tartrate was utilised by the three variants but β-hydroxybutyrate by types B and C only (see Experiments 35, 36). In sucrose peptone liquid culture only physical differences (e.g. pellicle formation and granulation) distinguished the types. No difference was observed in the action on litmus milk but acid was produced in/

in 2% purple lactose agar by type C only - the others showing no pH change during 12 days' incubation. Pigment production was the same in all three types.

These experiments indicate that, owing to the instability of some of the strains of Pseudomonas tested, little reliance can be placed on colony appearance as an aid to identification.

PART VI

The isolation of Pseudomonas from diseased plant tissue.

The isolation of Pseudomonas from diseased plant tissue.

Introduction

The isolation of *Pseudomonas* from diseased plant tissue does not normally cause much difficulty if the infection was recent. The maceration of a piece of the infected tissue in a small volume of sterile water, followed by a direct plating on a suitable nutrient agar is usually sufficient to provide the required colonies.

Under certain circumstances, however, the desired organisms are very few in number or are accompanied by large numbers of saprophytic fungi or bacteria. This is often found to be the case when examining the stem or branch cankers of trees infected by *Ps. mors-prunorum* or related organisms at a time when only a few viable cells remain.

The possession of a medium reasonably selective for the pseudomonads would thus be of considerable use. Specialised media for *Ps. phaseolicola* and for *Ps. mors-prunorum* have been described by Wilson (1938) and Crosse & Bennett (1955) respectively. The value of the latter medium relies on the observation that *Ps. mors-prunorum* is capable of utilising tartrate as the sole source of carbon. Di-octyl sodium sulphosuccinate ("Manoxol OT") is included in the medium for the suppression of saprophytes. Although Erikson (1945) found that not all strains of *Ps. mors-prunorum* utilise tartrate, Crosse & Bennett found no evidence that the/

the medium was selective for certain strains.

Experiment 35

Medium containing sodium tartrate as the source of carbon.

A selection of 204 pseudomonad strains of widely varied origin were inoculated into an ammonium salt medium containing 0.5% sodium tartrate. The cultures were incubated for 10 days at 27°C. Growth was normally accompanied by a marked alkaline reaction indicated by brom thymol blue.

Results

The results (Table 26) showed that 155 (77%) of the strains were capable of growing with sodium tartrate as the carbon source. These include a large proportion of the strains that may be presumed to be non-pathogenic. Five of the six mors-prunorum strains grew rapidly (48 hrs.) and one slowly (7 days) in the tartrate medium. Of the eleven syringae strains, three grew rapidly, six grew slowly and the two remaining strains produced no growth.

- - - -

Experiment 36

Medium containing sodium β-hydroxybutyrate as the source of carbon.

Introduction

Lemoigne, Girard & Jacobelli (1950) observed that when

a/

a medium containing sodium /3-hydroxybutyrate, as the only source of carbon, was inoculated with soil organisms the initial growth was almost invariably composed of Pseudomonas. Sodium aceto-acetate provided similar results (Villecourt, Blachère & Jacobelli (1952)).

When /3-hydroxybutyrate (0.5%) was used instead of tartrate in the medium of the previous experiment 177 (88%) of the 204 organisms tested grew with the production of an alkaline reaction. Further investigation of the results revealed that only a small proportion (approximately 6%) could use neither the tartrate nor the /3-hydroxybutyrate. (Table 26.) Two of the six mors-prunorum and seven of the eleven syringae strains grew rapidly (48 hrs.) in the medium. Most of the remainder grew slowly (7 days) while one syringae strain showed no growth after an incubation of 14 days.

All the strains of mors-prunorum and syringae grew satisfactorily in one or both of the tartrate and butyrate media.

Experiment 37

Medium containing sodium gluconate as the source of carbon.

The same series of strains was inoculated into the ammonium salt medium containing 0.1% sodium gluconate. As noted elsewhere (Experiment 4) all the test organisms grew in this medium. In view of this finding further trials were/

TABLE 26

Growth in an ammonium salt medium containing either sodium tartrate or sodium β-hydroxybutyrate as the sole source of carbon

Constituent of medium	Numbers showing growth
T or B	139
T	16
B	37
Neither	12

Key

T ... sodium tartrate

B ... sodium β-hydroxybutyrate

were made using soil suspensions and fragments of plant tissue as inocula. In almost every case pseudomonads were the first organisms to become dominant except when yeast like cells grew rapidly at the expense of the bacteria. This growth of the fungi could be prevented or delayed by adding di-octyl sodium sulphosuccinate (0.01%) as recommended by Crosse & Bennett (1955).

- - - -

The use of the selective media in practice.

The tartrate and /3-hydroxybutyrate media have been used for two or three years for enrichment purposes in the routine examination of diseased plant tissue (e.g., Clark & Paton, 1956). Portions of the tissue cut from the plant are used to inoculate both media. After 48 hrs. the resulting growths are plated on 5% sucrose agar and ordinary nutrient agar. The colonies are picked into a partially chelated gluconate medium (see Experiment 5), and the growth checked for fluorescence. Isolations carried out in this manner have rarely failed. There is, however, an obvious disadvantage in using two media for enrichment at each examination.

This work has been the subject of a paper read before a meeting of the Society for Applied Bacteriology and a summary was subsequently published (Paton, 1954).

The gluconate medium with "Manoxol" has also given satisfactory/

satisfactory results in practice but present experience of it has not been adequate to justify a comparison with the tartrate-butyrate method.

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PART VII

The control of the disease.

The control of the disease.

Introduction

The control of bacterial canker in Britain has depended largely on the use of resistant varieties and rootstocks. High-working and staddle-working on more resistant stocks has reduced the incidence of main stem canker. The use of Bordeaux mixture has been recommended for use in the case of the cherry but such sprays have proved less successful on the plum (Crosse, 1954).

The use of antibiotics for the control of plant diseases has been the subject of a number of investigations. Although most of these have been concerned with diseases caused by fungi a few refer to bacteria (e.g. Brian, 1952, 1954). Dye & Dye (1954) tested aureomycin, terramycin, chloromycetin, and streptomycin to determine their effect in preventing an infection of seedling peach trees by Ps. syringae under greenhouse conditions in New Zealand. The compounds were applied as foliage sprays before the plants were inoculated. They found that streptomycin was the most effective and gave a significant control without any phytotoxicity. The other antibiotics used in their experiment/

experiment were not so effective as streptomycin although they gave some degree of control at high concentrations. Subsequent preliminary field trials by one of the above workers (Dye, 1954[b]) in different seasons confirmed the ability of streptomycin to give a high degree of protection to peach seedlings.

The application of the antibiotic by means of a spray appears to be the only feasible method, at present, of ensuring an adequate and uniform treatment of trees. It has been shown by Pramer (1953, 1954) that streptomycin is absorbed by the root systems of beans, cucumber, and tomato seedlings, but the movement and accumulation of the antibiotic in the plants is very slow. No similar experiments have, apparently, been carried out with plum seedlings but it is very doubtful that an application of streptomycin to the roots of trees would be effective in practice as the antibiotic is very rapidly inactivated in soil (Jefferys, 1952).

As a result of the promising work carried out in New Zealand with a streptomycin spray an investigation was begun to determine the value of such a treatment under conditions prevailing in Britain.

Experiment 38 The testing of streptomycin applied in a spray.

A preliminary experiment in the laboratory showed that all/

all the available strains of Ps. mors-prunorum and Ps. syringae were sensitive to streptomycin. The tests were carried out by a cup-assay method on nutrient agar using streptomycin hydrochloride at concentrations of 50, 100 and 300 i.u./ml. Even at the lowest of these concentrations marked zones of inhibition appeared with all the strains tested.

A suitable site was offered at Melrose, where an orchard had been established for about 10 to 15 years. Since the original planting, losses, due mainly to canker, have made many replacements necessary. The orchard was thus found to be composed of trees of a variety of ages. As a result of a survey, 111 trees which had been in situ for three years or more were selected for an experiment designed to test a streptomycin spray. With the co-operation of Glaxo Laboratories Ltd., the layout of the trial was planned to give the maximum amount of information regarding the efficacy of the treatment (Orchard plan "A"). This scheme provided for a spray treatment of 40 trees each month from November to April inclusive. Of these, 16 were treated each month over the whole period while 24 trees were treated only in the first three months and another 24 in the second three months of the trial. The untreated control was made up of 47 trees. This programme took into account the two critical infection periods, viz., the autumn infection/

Orchard Plan A

"The Croft" Melrose, Roxburghshire.

Distribution of trees in streptomycin spray trial

Column / Row	10	9	8	7	6	5	4	3	2	1
A	1	28	29	56	-	74	75	94	95	-
B	2	27	30	55	57	73	76	93	-	111
C	3	26	31	54	-	72	77	92	96	110
D	4	25	32	53	58	71	-	91	-	109
E	5	-	-	52	59	-	78	90	-	108
F	6	24	33	51	-	-	79	-	97	-
G	7	-	34	50	60	-	-	-	98	-
H	8	-	35	-	-	70	-	89	99	107
I	9	-	36	49	61	69	-	88	100	106
J	10	23	37	48	-	-	-	87	101	-
K	-	-	38	47	62	-	-	86	102	-
L	-	22	-	-	-	-	80	85	-	105
M	11	21	39	46	63	-	-	-	-	104
N	-	20	-	45	-	-	81	-	-	103
O	12	19	-	44	64	-	-	-	-	-
P	-	18	-	43	65	68	-	84	-	-
Q	-	17	-	-	66	-	82	-	-	-
R	-	16	-	-	-	-	83	-	-	-
S	-	15	-	42	67	-	-	-	-	-
T	-	14	-	-	-	-	-	-	-	-
U	-	13	40	-	-	-	-	-	-	-
V	-	-	-	41	-	-	-	-	-	-
Treatment	S_2	C	S_2	S_1	C	S_3	S_3	C	S_3	C

Treatments

S_1 & S_2 monthly in Nov., Dec., & Jan. (40 trees)

S_1 & S_3 monthly in Feb., March, & Apr. (40 trees)

C control.

The blank areas contain trees which, for various reasons, were not included in the trial.

infection of the bark, and the spring infection of the first leaves (see page 4).

The materials were provided by Glaxo Laboratories Ltd., in the form of two free-flowing powders (a wetting-out agent and streptomycin hydrochloride) which only required to be mixed with water to form a relatively stable solution. The active streptomycin content of the spray, as applied to the trees, was 220 i.u./ml. One complete spraying programme was carried out using this preparation but, in the following programme (1955-56), a slight modification was made. Instead of the two powders, mentioned above, a concentrated liquid containing both the streptomycin and the wetting-out agent was used. The streptomycin content of the prepared spray was similar.

The spraying was carried out on each occasion in still, dry weather in the absence of frost by means of a knapsack outfit operated by two persons. An average of 8 gal. of spray was required for each operation involving 40 trees; a slightly larger volume being required when the trees were in leaf.

Protective clothing was worn by the operators to avoid, as far as possible, wetting the skin with the antibiotic. This was thought to be a reasonable precaution as streptomycin has been known to cause a severe dermatitis in sensitive individuals.

The co-operation of a hospital laboratory was obtained for/

for an investigation of the effect of the spray on the bacterial flora of the operators. An examination was made of the organisms of the nose, throat, hands, and intestinal tract over a period of several months. No evidence was forthcoming that ~~the~~ streptomycin-resistant strains had appeared.

Results

This trial is now, (Spring, 1956), in its second year. No information of value was obtained from the first year's treatment as no canker appeared on any of the trees. This was perhaps due largely to the fact that the season was abnormally dry. It is interesting to note that in the Clyde valley during the same season canker appeared to be as destructive as usual. The second of the annual spraying programmes is still progressing.

Experiment 39 The testing of streptomycin paint.

Introduction

Elsewhere (p. 4) it was noted that a large proportion of main stem cankers apparently originated from an infection of the bark at the point of tying. The necessity of staking and tying young fruit trees in exposed orchards is obvious. Various types of "tie" have been marketed but none avoids a certain amount of abrasive action on the bark. Some growers use strips of rubber (cut from the old inner tubes of cars) bound around the stem and then fastened to the stakes/

stakes. These do not wear down the bark to the same degree but have the disadvantage of retaining moisture and of sheltering micro- and macro-organisms, and are not generally recommended.

Experimental

The use of streptomycin again suggested itself as a possible solution to the problem. Mitchell, Zaumeyer and Anderson (1952) have shown that streptomycin applied in lanolin to stems of Phaseolus confers resistance to the leaves against bacterial blight caused by Ps. medicaginis. A paint, again formulated by Glaxo Laboratories Ltd., was produced which contained streptomycin in a plastic emulsion base. This preparation had a streptomycin potency of 10,000 i.u./ml. When painted on areas of bark subject to abrasive action it formed a protective layer acting both as a mechanical and a chemical barrier against infection.

This "paint", when exposed in the open air, was found to retain its antibiotic activity for approximately 6 months. The areas of bark subject to abrasion were, however, repainted every 3 months.

Using this method an experiment was set up in 1954 to test its value in the prevention of canker.

The site chosen for the experiment was part of a walled-garden at Dryden near Edinburgh. Victoria plum trees of considerable age grew at regular intervals on the inside of the wall. These trees suffered from bacterial canker/

canker and were considered to be a good source of infection in the garden. This offered a good opportunity to test out the streptomycin paint and consequently 100 trees direct from a nursery were planted within the garden. These were so arranged that 25 blocks of 4 trees were formed (Orchard plan "B"). Each of the 4 trees in each block was subjected to a different treatment (randomized) viz.,

1 ... Inoculated, unstaked and unpainted.

2 ... Staked, painted and uninoculated.

3 ... Control.

4 ... Staked, unpainted and uninoculated.

The inoculations were carried out using a suspension of 3 strains, (one of Ps. mors-prunorum and two of Ps. syringae) in semi-solid gelatin. Holes were made in the bark with a cork-borer at points where the trees are normally tied; these were filled with the suspension and covered temporarily with cellulose tape to prevent rapid drying. The object of using gelatin was to provide a slow but continuous flow of organisms into the wound as the gelatin was liquefied.

Results

As in the previous experiment no results are yet available.

Orchard Plan "B"

Dryden Garden, Midlothian

Distribution of trees in streptomycin in paint trial

Block									Block
A	2	1	4	3	2	3	1	4	Y
B	3	4	1	2	4	2	3	1	X
C	1	4	3	2	4	2	1	3	W
D	2	3	1	4	1	3	4	2	V
E	2	1	4	3	2	3	4	1	U
F	3	1	2	4	3	2	4	1	T
G	1	4	3	2	2	4	3	1	S
H	3	2	1	4	2	4	3	1	R
I	1	4	3	2	3	2	1	4	Q
J	1	2	4	3	1	2	4	3	P
K	3	2	4	1	4	2	1	3	O
L	4	1	2	3	3	2	1	4	N
					2	3	4	1	M

Key

Each block (A - Y) contains 4 trees (1 - 4).

Each of the 4 trees in each block represents a different treatment, viz.,

1 ... inoculated, unstaked and unpainted.
2 ... staked, painted, and uninoculated.
3 ... control.
4 ... staked, unpainted and uninoculated.

The trees are 15 feet apart.

DISCUSSION and SUMMARY

DISCUSSION

The close relationship of Ps. mors-prunorum and Ps. syringae noted in the literature is confirmed by the results described above; only minor differences have been detected. For example, in Experiment 13, mors-prunorum produced a pH change in a shorter time than syringae in the higher ratios of the lactose-peptone medium. Such differences, even if they are proved to be consistent, must be considered of minor practical value in differentiating these organisms.

From the evidence provided by the limited number of each species available it is obvious that the various strains of mors-prunorum produce very similar reactions (Table 27). The series of syringae strains is not so uniform. None of the characters distinguishes them specifically from the strains of mors-prunorum or, indeed, any other of the pathogens included in this study. A number of the characters are common to both species and three of the strains of syringae appear identical with mors-prunorum. This finding supports the opinion of Erikson (1945) that these organisms do not merit specific rank.

It is interesting to note here that Wormald (1938) isolated Ps. mors-prunorum not only from the plum but also from related hosts, e.g. Morello cherry, cherry plum, peach, and ornamental almond. Elliott (1951), in a description of Ps./

Ps. syringae, gives a list of its hosts which includes species of many different plant genera in addition to Prunus. Little evidence is available on the cross-pathogenicity of Ps. mors-prunorum. Erikson, (1945), however, observed that the majority of the isolates designated by her as Ps. mors-prunorum and also other strains derived from apricot, pear and syringa produced infections of dwarf bean pods on at least one of the three occasions on which they were tested. Crosse (1953) also found evidence of cross-pathogenicity in strains of Ps. mors-prunorum. In a description of blast of stone-fruit trees in New Zealand, Dye (1954[a]) found that the symptoms of the disease agreed with those attributed to both Ps. mors-prunorum and Ps. prunicola. It appears, therefore, that even when the host-pathogenicity of the organisms and the symptoms of the disease are considered there is little to distinguish them.

When the results of a number of experiments are analysed according to the source of the strains a noticeable division is apparent between the organisms isolated from plants and those from other sources (Table 28). The division, although not complete, was sufficiently clear to be considered significant. The fact that an organism is isolated from a particular source does not prove that source to be its normal habitat. This is particularly true of plants since on them soil organisms frequently occur/

occur. This possibility may account in some measure for the incomplete separation according to source. The suggestion is supported by the observation that the characters of the pathogens show an even greater contrast with those of the soil isolates. It is suggested, therefore, that the plant pathogens of this genus should not be considered as a circumscribed group but as specialised members of a larger group of Pseudomonas associated with plants. This group possesses, with a few exceptions, the following characters:-

(a) The utilisation of sucrose with or without the production of acid.

(b) The formation of levan from sucrose.

(c) Copper-reducing substances not produced from gluconate.

No adequate information has been forthcoming in this work by means of which the pathogens of stone-fruit trees can be distinguished from this general plant group.

TABLE 27

A comparison of strains of Ps. mors-prunorum and Ps. syringae

The following are details of the reactions relative to the accompanying disucssion. The strains referred to were all obtained from reliable sources as stated on page 8 of this study.

Strains	Utilisation of sucrose as sole carbon source (Expt. 14)	Acid in "purple lactose" agar (Expt. 13)	Copper-reduction test on sucrose broth. (Expt. 15)	Formation of levan on sucrose agar (Expt. 16)	Action on litmus milk. (Expt. 16)	Growth in tartrate medium (Expt. 36)	Growth in β - hydroxy--butyrate medium. (Expt. 36)	Lipolytic activity. (Expt. 27)
Ps. mors-prunorum								
1	X	X	X	X	cl	X	X	-
2	X	X	X	X	cl	X	X	-
3	X	X	X	X	cl	X	X	-
4	X	X	X	X	cl	X	X	-
5	X	X	X	X	cl	X	X	-
6	X	X	X	X	cl	X	X	-
Ps. syringae								
1	X	X	X	X	cl	X	X	-
2	X	X	X	X	cl	X	X	-
3	X	-	X	X	cl	-	X	-
4	X	-	X	X	cl	X	X	-
5	X	-	X	X	CD	X	X	-
6	-	X	-	-	cl	X	X	-
7	X	X	X	-	cl	X	-	-
8	X	X	X	X	cl	X	X	-
9	X	-	X	X	cl	X	X	-
10	X	-	X	X	cl	X	X	-
11	-	-	-	-	cl	-	X	X

Key

cl ... clearing without formation of curd.

CD ... curd with subsequent digestion.

The following characters were common to all of the above strains:-

a) no gas formation from nitrate (Expt. 28).
b) no lecithinase activity (Expt. 30).
c) no haemolytic activity (Expt. 31).
d) production of a water-soluble fluorescent pigment (Expt. 5).

TABLE 28

The analysis of reactions of the strains according to their source

Reaction	Isolations (including authentic pathogens) from plants.	Authentic pathogens.	Isolations from soil.	Expt.
Acid in				
sucrose-salts	58	86	61	14
Sucrose-peptone	46	86	2	14
Sucrose-tryptone	68	91	30	14
Copper-reduction in sucrose cultures	57	92	11	15
Formation of levan from sucrose.	68	86	24	16

SUMMARY

The object of the work reported here was threefold, the aim was, firstly, to examine the relationship of two species of bacteria pathogenic to stone-fruit trees, namely, Pseudomonas mors-prunorum Wormald and Pseudomonas syringae van Hall; secondly, to compare these organisms and some other pathogens with a series of pseudomonads isolated from plants and soil, with a view to improving the methods by which the pathogens may be isolated and identified; and thirdly, to investigate a possible means of controlling the disease on stone-fruit trees.

A brief account is given of the nature and incidence of bacterial canker of plum trees in Scotland.

A procedure for chelating heavy metals in culture media was found to promote the appearance of the fluorescent pigment produced by the pseudomonads. Traces of iron in the media were shown to interfere with the production of the pigment. A medium was described whereby the pigment was more readily detected.

The action of the organisms on various sugars was investigated. No significant differentiation was found on the basis of acid production from sugars but the utilisation of sucrose as the sole source of carbon was considered to be a significant differential character.

Levan/

Levan production from sucrose and the copper-reducing properties of sucrose cultures were studied. The two properties, although both were often found in one organism, were shown to be independent of each other.

An investigation was made of the production of 2-keto-gluconate from glucose and related compounds. The majority of the plant pathogens tested did not produce the keto-acid which was, however, commonly a product of the other organisms.

The lipolytic activity of the organisms was examined using an improved method for its detection. This property was found to be less common among the plant pathogens than the other organisms tested.

Observations on the stability of the colonial characters led to the conclusion that little reliance can be placed on the features of the colony as an aid to the identification of the organisms.

The strains of Ps. mors-prunorum examined in this work could not be distinguished, by the laboratory methods applied, from the strains of Ps. syringae. The grounds on which these species are separated appear, therefore, to be questionable. In the whole series of pseudomonads examined the laboratory examination did bring out one line of division which could be related to the sources of the organisms: the strains isolated from plants (including the pathogens) could be separated from those from the soil by the possession of the following characters by the plant organisms,/

organisms, viz.,

(a) The utilisation of sucrose with or without the production of acid.

(b) The formation of levan from sucrose.

(c) No copper-reducing action from gluconate.

In view of this finding it may be suggested that the plant pathogens in this genus may be regarded as more or less specialised members of a larger group of Pseudomonas, which normally live in association with plants.

Work was done on methods of isolating pseudomonads from diseased plant tissue. Of the techniques tried the most promising was one involving the use of sodium gluconate as the sole source of carbon.

The information derived from the above studies was of assistance in the conduct of two field experiments. These were designed to test the value of streptomycin in the control of bacterial canker of stone-fruit trees. In one the streptomycin is applied as a spray with the object of destroying organisms on the external surfaces of the trees. In the other experiment, in which the streptomycin is applied as a paint, the object was to prevent infection where the bark was subject to abrasion.

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