https://theses.gla.ac.uk/

Theses Digitisation:

https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses
https://theses.gla.ac.uk/
research-enlighten@glasgow.ac.uk

UNIVERSITY OF GLASGOW

TAXONOMIC STUDIES OF MICROCOCCI AND STAPHYLOCOCCI

by

EWEN CAMERON DAVID TODD, B.Sc.

Thesis presented for the degree of Doctor of Philosophy
in the Faculty of Medicine.

Department of Microbiology February, 1968.

ProQuest Number: 10647049

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 10647049

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

GLASGOW
UNIVERSITY
LIBRARY

"all things began in order, so shall they end, and so shall they begin again"

Cyrus' Garden

Sir Thomas Browne.

SECTION A

INTRODUCTION

AND REVIEW OF THE

LITERATURE

C O N T E N T S

SECTION A

Page

INTRODUCTION AND REVIEW OF THE LITERATURE

INTRODUCTION AND REVIEW OF THE LITERATURE

INTRODUCTION 1
THE FAMILY MICROCOCCACEAE 6
GENERA WITHIN THE FAMILY MICROCOCCACEAE -
The genera *Micrococcus* and *Staphylococcus*. 12
The genus *Sarcina* 15
The genus *Gaffkya* 16
The genus *Sporosarcina* 18
The anaerobic genera 18
The species of the genus *Micrococcus* -
Micrococcus luteus 19
Micrococcus roseus 20
Other *Micrococcus* species 21
The species of the genus *Staphylococcus* -
Staphylococcus aureus 22
Other *Staphylococcus* species 23
Comments on the definitions of specific names of the genus *Staphylococcus* by subsequent taxonomists 23
CHARACTERS USED IN TAXONOMIC STUDIES OF MICROCOCCI AND STAPHYLOCOCCI 26
MORPHOLOGICAL CHARACTERS -
Shape of the Cells 30
Size of the Cells 30
Motility of the Cells 31
Colonial Form of the Cells 31
Appearance of Cells in Broth Culture 32
Gram's Reaction of Cells in Stained Smears 32
PHYSIOLOGICAL CHARACTERS -
The Optimum Temperature for Incubation of Cultures 33
The use of Temperature Growth Range in Classification 34
Resistance of Organisms to Heat 34
Sodium Chloride Resistance 35
Prototroph Activity - Growth in Glucose. Ammonium Phosphate Medium 36
Pigmentation 37
Utilisation of Carbohydrates 39
Production of Acetoin 44
Nitrate Reduction 46
Catalase Production 46
Oxidase Activity 47
Proteolytic Action 49
Hydrolysis of Urea 51
Lipase Activity 52

SECTION A

		Page
REVIEW OF THE LITERATURE (contd.)	Coagulase Production	53
	Phosphatase Production	55
	Haemolysin Production	56
	COMPARISON OF THE MAIN MORPHOLOGICAL AND PHYSIOLOGICAL CLASSIFICATION SCHEMES OF MICROCOCCI AND STAPHYLOCOCCI FROM 1948 TO 1965	60
	SEROLOGICAL CHARACTERS	64
	PHAGE TYPING CHARACTERS	65
	ECOLOGICAL CHARACTERS	68
	THE USE OF EXTRACTABLE CELL COMPONENTS AS CHARACTERS FOR CLASSIFICATION -	
	Cell Wall Sugars, Amino Sugars and Amino Acids	71
	Relative Molecular Proportions of Cell Wall Amino Acids	73
	Teichoic Acids	75
	Acetic Acid-Extractable Amino Acids and Peptides from Whole Cells	76
	THE MOLECULAR BIOLOGICAL APPROACH TO CLASSIFICATION -	
	DNA Base Ratios	78
	Nearest Neighbour Base Sequence Analysis of DNA	86
	DNA Homology	86
	THE ADANSONIAN APPROACH TO CLASSIFICATION	89
MATERIALS AND METHODS	INTRODUCTION TO EXPERIMENTAL WORK	94
	STRAINS OF MICROCOCCI AND STAPHYLOCOCCI	
	Origin of Strains	96
	Isolation of Strains	96
	Maintenance of Cultures	98
	EXAMINATION OF MORPHOLOGICAL CHARACTERS	99
	EXAMINATION OF PHYSIOLOGICAL CHARACTERS -	
	The optimum temperature of incubation	100
	Pigmentation	100
	Sodium chloride resistance	100
	Catalase production	101
	Oxidase production	102
	Production of acid from carbohydrates	102
	-galactosidase activity	105
	Starch hydrolysis	106
	Growth in glucose ammonium phosphate medium	106
	Acetoin production	107
	Coagulase production	108
	Phosphatase production	111
	Proteolysis	111
	Urea hydrolysis	112

SECTION A

		Page
MATERIALS AND METHODS (contd.)	Lipolysis	112
	Haemolysin production	113
	Phage typing	114
	PHOTOGRAPHY	114
RESULTS	INTRODUCTION TO THE RESULTS' SECTION	115
	Cell size and cell shape	116
	Gram's reaction of cells in stained smears	116
	Cell arrangement, colonial appearance of cells on agar and the form of growth in broth culture	116
	Motility of cells	117
	The optimum and critical temperatures for growth	117
	Pigmentation	118
	Growth on various concentrations of sodium chloride in agar	119
	Catalase production	121
	Oxidase production	121
	Utilisation of carbohydrates	
	The production of acid from glucose and mannitol	123
	Lactose utilisation and the ONPG test	133
	Starch hydrolysis	136
	Growth in glucose ammonium phosphate medium	137
	Acetoin production	138
	Coagulase production	141
	Phosphatase production	142
	Milk clearing and hydrolysis	143
	Gelatin hydrolysis	144
	Urea hydrolysis	144
	Tributyrin hydrolysis	145
	Egg yolk opacity	145
	Haemolysin production	147
	Phage typing	151
	ECOLOGICAL CHARACTERS	153
DISCUSSION OF RESULTS	INTERESTING FINDINGS	154
	THE SIGNIFICANCE OF MAIN CHARACTERS IN A CLASSIFICATION SCHEME OF MICROCOCCI AND STAPHYLOCOCCI	157
	Catalase production	158
	The utilisation of carbohydrates	160
	Coagulase production	170
	Phosphatase production	171

SECTION A

Page

DISCUSSION OF RESULTS (contd.)

Pigmentation 173

THE ADANSONIAN CLASSIFICATION SCHEME OF THE 406 STRAINS BASED ON MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERS 176

SECTION B

REVIEW OF THE LITERATURE

ELECTROPHORESIS AS A TAXONOMIC TOOL

The technique of electrophoresis 181

The use of zone electrophoresis in classification of bacteria 183

Criticism of the applications of zone electrophoresis for the classification of strains in the family *Micrococcaceae*.. 183

MATERIALS AND METHODS

PREPARATION OF CELL CONTENTS FOR ELECTROPHORESIS

Batch culture of cells

Method 1 - Large scale broth culture 186

Method 2 - Agar culture of cells 190

Disintegration of cells 193

The removal of insoluble particles from the disintegrates 194

Storage of cell contents 195

ELECTROPHORESIS OF CELL CONTENTS

Preparation of gel support media for electrophoresis 195

Starch gel 196

Acrylamide gel 197

Electrophoresis of cell contents on starch and acrylamide gels 200

Slicing of gels 201

METHODS FOR DETECTING PROTEIN AND ENZYMES IN GEL SLICES 202

PROTEIN STAIN 203

ESTERASE STAIN 203

ACID PHOSPHATASE STAIN 206

-GLUCURONIDASE STAIN 208

PROTEOLYTIC ENZYME DETECTION 209

UREASE DETECTION 211

LIPASE DETECTION 212

SUCCINIC DEHYDROGENASE STAIN 214

SECTION B

		Page
MATERIALS AND METHODS (contd.)	LACTIC DEHYDROGENASE STAIN	214
	MALIC DEHYDROGENASE STAIN	215
	PEROXIDASE STAIN	215
	CATALASE DETECTION	216
	CYTOCHROME OXIDASE STAIN	217
	MONAMINE OXIDASE STAIN	218
	DOPA OXIDASE STAIN	218
	STARCH HYDROLYSING ENZYME DETECTION	219
	BLOOD BAND DETECTION	219
	THE EFFECT OF HEAT ON ISOZYMES	220
	THE EFFECT OF CHEMICAL INHIBITORS ON ISOZYMES	221
	PHOTOGRAPHY	222
	APPENDIX	223
RESULTS	THE RESULTS OF THE ELECTROPHORETIC ANALYSIS OF CELL CONTENTS OF 406 STRAINS OF MICROCOCCI AND STAPHYLOCOCCI	224
	THE DETECTION OF PROTEIN AND ENZYME BANDS IN GEL SLICES -	
	PROTEINS	225
	ESTERASE ISOZYMES	226
	ACID PHOSPHATASE ISOZYMES	231
	-GLUCURONIDASE ISOZYMES	233
	PROTEOLYTIC ISOZYMES	233
	UREASE ISOZYMES	234
	LIPOLYTIC ISOZYMES	240
	SUCCINIC DEHYDROGENASE ISOZYMES	242
	LACTIC DEHYDROGENASE ISOZYMES	244
	MALIC DEHYDROGENASE ISOZYMES	246
	PEROXIDASE ISOZYMES	246
	CATALASE ISOZYMES	247
	CYTOCHROME OXIDASE ISOZYMES	250
	MONAMINE OXIDASE ISOZYMES	250
	DOPA OXIDASE ISOZYMES	251
	STARCH HYDROLYSING ISOZYMES	252
	BLOOD BAND ISOZYMES	256
	THE SIGNIFICANCE OF HEAT AND ENZYME INHIBITORS IN THE CLASSIFICATION OF ESTERASES -	
	Heat	263
	Chemical enzyme inhibitors	265
	HEAT RESISTANCE AND INHIBITION EXPERIMENTS ON PEROXIDASES, CATALASES AND BLOOD BANDS -	
	Heat	268
	Chemical enzyme inhibitor	269
	COMPARISON OF BANDS -	

SECTION B

Page

RESULTS (contd.)

COMPARISON OF BANDS (contd.)

The relationship between high running bands produced by Peroxidase Methods 1 and 2 and those produced by Succinic Dehydrogenase Method 1 and the Monamine Oxidase Method 272

The relationship between bands produced by Peroxidase Method 1, Catalase Detection Method and Blood Band Detection Method ... 273

SUMMARY 274

THE ELECTROPHORETIC CLASSIFICATION SCHEME - ADANSONIAN CLASSIFICATION OF THE 406 STRAINS OF MICROCOCCI AND STAPHYLOCOCCI BASED ON ELECTROPHORETIC CHARACTERS - ESTERASE, BLOOD BAND, AND STARCH HYDROLYSING ISOZYMES 277

DISCUSSION

DISCUSSION OF THE ELECTROPHORETIC GROUPS

The taxonomic significance of cell shape in the genus *Micrococcus* 305

The taxonomic significance of catalase production in the genus *Micrococcus* 306

Comparison of Electrophoretic *Micrococcus* Groups with morphological and physiological groupings of micrococci and staphylococci by other taxonomists 308

Comparison of ecological characters with the Electrophoretic Groups 311

Comparison of Electrophoretic *Micrococcus* Groups with published classification schemes based on DNA base ratios 313

The use of the electrophoretic classification scheme for identifying micrococci and staphylococci 314

SECTIONS A & B

SUMMARY 316

BIBLIOGRAPHY 320

TABLES

	Between pages or on page
Table 1	11 - 12
Tables 2, 3, 4 & 5	22 - 23
Table 6	30 - 31
Table 7	34 - 35
Table 8	35 - 36
Table 9	37 - 38
Table 10	40 - 41
Table 11	52 - 53
Table 12	60 - 61
Table 13	61 - 62
Table 14	64 - 65
Table 15	69 - 70
Table 16	72 - 73
Table 17	73 - 74
Table 18	75 - 76
Table 19	79 - 80
Table 20	80 - 81
Table 21	81 - 82
Table 22	90 - 91
Table 23	97 - 98
Table 24	117
Table 25	118
Table 26	120
Table 27	122
Table 28	128
Table 29	136
Table 30	140
Table 31	143
Table 32	144
Table 33	146
Table 34	148
Table 35	150

	Between pages or on page
Table 36	160
Table 37	171 - 172
Table 38	183 - 184
Table 39	236
Table 40	238
Table 41	249
Table 42	253
Table 43	264
Table 44	266
Table 45	268
Table 46	270
Table 47	272
Table 48	313 - 314

DIAGRAMS

Diagram I	187 - 188
Diagram II	188 - 189
Diagrams III & IV	197 - 198
Diagram V	224 - 225
Diagram VI	244 - 245
Diagram VII	273 - 274

PLATES

Plate 1	225 - 226
Plates 2, 3 & 4	226 - 227
Plate 5	248 - 249
Plate 6	252 - 253
Plate 7	256 - 257
Plate 8	257 - 258
Plates 9 & 10	275 - 276

CLASSIFICATION SCHEMES

Scheme 1	pocket inside front cover
Scheme 2	pocket inside back cover

INTRODUCTION

In my review of the literature I shall discuss the taxonomy of the family *Micrococcaceae*. Unfortunately, in the literature the words "taxonomy" and "classification" are frequently considered to be synonymous. I, however, am not of this opinion. I define taxonomy as the science of the arrangement, the naming, and the identifying of living things, and classification as the arrangement of living things into groups or classes. I consider, therefore, like Cowan (1965), that taxonomy can be divided into 3 parts (1) classification, (2) nomenclature, and (3) identification. Classification is the most important part of taxonomy, since without a proper classification scheme, neither the naming nor the identifying of an organism can be carried out satisfactorily; unfortunately the earlier taxonomists did not realise the importance of a classification scheme based on valid and reproducible criteria and this has led to the considerable confusion of the taxonomy of the family *Micrococcaceae* in the literature.

Each of the 3 components of taxonomy has its own particular problems, but it is not always possible to deal with the problems of classification, nomenclature, or identification in isolation from one another. This is why, to the bacteriologist in general, classification, nomenclature and identification often appear to be fused together. The critical taxonomists, therefore must try and separate these 3 components when he reviews

the literature, and also presents his own contribution to the science of taxonomy.

1. Classification

(a) There must be a purpose for classifying strains of bacteria. My reason for classifying strains of the family *Micrococcaceae* is to show, whether, within this large group of organisms, strains can be regarded only as a part of a continuous series with pathogenic, biochemically active coagulase positive strains at one extreme and saprophytic, biochemically weak or inactive, coagulase negative strains at the other, or whether any natural groupings exist.

(b) A large variety of characters must be used. All classification schemes of bacteria are based on the comparison of certain characters exhibited by strains of these bacteria. In any taxonomic study, therefore, it is important to examine as many characters as possible in order to show relationships between strains. I have used morphological and physiological characters, as both of these have been shown to be the most valuable in previous taxonomic studies for the classification of micrococci and staphylococci; I have also used characters detected by electrophoretic methods, e.g. isozymes of cell distintegrates, which have not been used in previous classifications of this group of organisms. Cell composition characters, e.g. DNA base ratios, are well worth examining but they are a study in themselves and

were not used in this classification. I have not used serological characters as a means of division of the aerobic members of the Micrococcaceae, since previous work has shown that these have been found to be of limited use only in one group, the Staphylococcus aureus group, within this family.

The taxonomic significance of ecological characters, such as habitat, in the Micrococcaceae, however, has not, up till now, been fully studied. Therefore, habitat characters have been examined to see if they correlate well with any other set of characters.

(c) Weighting of characters

Once the characters of strains have been examined there are two ways to try to classify the strains.

(1) The traditional approach to classification - In the traditional approach to classification, individual taxonomists select certain characters they believe to be important, as criteria for the grouping of strains into defined families, genera, species or groups. These arbitrarily chosen divisions may suit the individual taxonomists and those interested in their approach to classification, but this approach does not show a true relationship between strains.

(2) The Adansonian approach to classification - Since little information is known about the importance of characters, one character should not be emphasised more than another for the

arrangement of strains into families, genera, species or groups, and thus in an Adansonian classification each character is given equal weight. I have adopted the Adansonian approach to my classification schemes, since it is the only way natural groupings can be detected.

2. Nomenclature

As a result of any taxonomic study, names are applied to each taxonomic unit to give precise, although limited, information about each unit so that communication can be readily made between people when referring to these units. The literature, however, is burdened with many binomial names which are either synonyms, or convey, by definition, very little information so that the nature of the taxonomic unit is vague.

Many taxonomists have rejected the idea of using names, which only confuse the reader and they prefer to use their own group and subgroup numbers; Cowan (1965) has actually advocated the complete abandonment of names and he recommends the use of a code for all bacteria consisting of numbers or letters. I have used binomial and other names to designate strains only when I have defined them first, and for certain natural groupings I use my own group or subgroup number.

3. Identification

Identification of bacteria is, of necessity, dependent on an earlier classification, and it is important that identification is a rapid and relatively simple standard procedure, since many

people who wish to identify bacteria are not taxonomists. One of the best ways to identify bacteria is to prepare a key based on a few selected characters which are typical of the natural groups found by classification schemes. The problem of identifying strains of the family Micrococcaceae is that few natural groups have been discovered, and therefore typical characters do not exist, which makes the construction of a simple diagnostic key impossible. At the moment, within this group of bacteria, identification has to be carried out by following classification schemes which depend on many tests for the examination of characters. I have discussed, within this thesis, the usefulness of an identification scheme based on my system of classification.

The following review deals with, first, the state of confusion existing in the taxonomy of micrococcal and staphylococcal strains; secondly, the usefulness of characters as taxonomic criteria for arbitrary classification schemes; and thirdly, the advantages, if any, of the Adansonian approach to classification over the traditional arbitrary one in the micrococcus-staphylococcus group.

THE FAMILY MICROCOCCACEAE

Goodsir had recorded the presence of coccal forms of bacteria from stomach contents in 1842 but it was thirty years later before any taxonomic study of cocci was attempted. Cohn (1872) was the first person to place spherical bacteria, which he had isolated from a variety of habitats including slime and wound secretions, into a genus - *Micrococcus*. It is clear from Cohn's description, however, that some of his isolates were diptheroids and streptococci. Six years later Magnin (1878) included both the genera *Micrococcus* and *Sarcina* in a bacterial classification scheme, but he did not make clear the relationship between the two genera, and it was not until 1883 that a true classification of spherical bacteria was made; Zopf (1883) put the genus *Micrococcus* and the genus *Sarcina* with three other genera, *Streptococcus*, *Merismopodia*, and *Ascococcus*, into one family, the *Coccaceae*, the original definition of which was as follows: "spherical elements, no spores, division in one or several planes" (Zopf - 1883, 3rd edition of Bergey's Manual, 1930). The spherical organisms were classified into five genera according to their microscopic morphological arrangement, i.e. various planes of division and zooglea formation; the genus *Micrococcus* was defined as spherical elements occurring in irregular masses, and the genus *Sarcina* was defined as spherical elements occurring in packets.

The word staphylococcus was first used by Ogston in 1882 in

describing cocci present in septic wounds; he also showed that these cocci were pathogenic to mice and guinea pigs. Ogston, however, used the word as a common name and not as a scientific one and it was Rosenbach (1884) who introduced Staphylococcus as a generic name into bacteriological nomenclature. The genus Gaffkya was created by Trevisan in 1885 in a little known classification scheme to cover tetrad forming micrococci; this generic name was ignored by contemporary taxonomists and was not used for another 48 years (Prévot, 1933). In 1886, the taxonomic work of Hueppe resulted in the earliest record of the three genera, Micrococcus, Sarcina and Staphylococcus being placed in the same family - the Coccaceae, but in fact it was 31 years later before these three genera were once more under the same family name (Buchanan, 1917). Although a few more bacterial classification schemes were evolved in the next 14 years (e.g. Flugge, 1886, Lehmann & Neumann, 1896, Migula, 1900), the number of characters used for classifying the Coccaceae into genera was not extended much beyond the few morphological ones of Zopf (1883) from the years 1886 to 1908.

It was of great significance, therefore, when Winslow & Winslow in 1908 emphasised for the first time the importance of Gram stain, pigmentation, the breakdown of sugars and the ability to cause disease as characters for the classification of the Coccaceae into genera. They defined the genus Micrococcus as being "generally Gram negative, irregularly grouped cells, usually

yellow pigmented, saprophytic and not able to produce acid from sugars;" the definition of Sarcina differs from that of Micrococcus only in that cells in Sarcina occur in packets. The definition by Winslow & Winslow (1908) of two other genera, differing only in pigmentation, was given as follows:- orange-pigmented Aurococcus and white-pigmented Albococcus - usually Gram positive irregularly grouped cells, parasitic and able to produce acid from sugars. In the light of present knowledge it is probable that the two genera Aurococcus and Albococcus correspond to the genus Staphylococcus Rosenbach.

Although Zopf had discovered a red pigmented coccus which he named Rhodococcus in 1891, it was Winslow & Winslow (1908) who introduced this genus for the first time into a classification scheme of the Coccaceae, and they differentiated it from the genus Micrococcus solely on the production of a red pigment. The genus Rhodococcus was recognised by Bergey in the first four editions of Bergey's Manual (1923, 1925, 1930 and 1934) and by a few taxonomists since, e.g . Kluyver & van Niel (1936), Rahn (1937), but most modern taxonomists now regard pink pigmented micrococci as being species in the genus Micrococcus.

The 25 years following the work of the Winslows (1908) have shown that the taxonomists of that time were not certain of the reliability of the Gram stain, at least within the family Coccaceae. For whereas Buchanan in 1917 and later in 1930

agreed with the results of Winslow & Winslow (1908), in that species of the genus Staphylococcus are usually Gram positive and that species of the genus Micrococcus are usually Gram negative, Hucker (1924a,b.) found that when he stained 316 strains of the genus Micrococcus by five different methods (all called Gram stain) he obtained completely different results. Bergey in his Manual (3rd Edition, 1930) and Janke (1930) considered that both the genera Staphylococcus and Micrococcus could be defined as generally Gram positive. Rahn (1937) posed the question that these varying opinions might be brought about by a change in the composition of the dyes since 1908.

In 1929 Pribram replaced the family name Coccaceae by a new name Micrococcaceae because he stated that Article 21 of the code of Botanical Nomenclature specified that family names are to be "designated" by the name of one of their genera or ancient generic names"(Committee of the Society of American Bacteriologists, 1917) and hence he considered that the name Coccaceae was illegitimate. Four years later the significance of the Gram stain in this group of organisms was ended once and for all when Pribram (1933) completely re-defined the family Micrococcaceae to include Gram positive cocci, occurring singly, in pairs, tetrads, packets, irregular masses or even in short chains. Although this definition is a broad one in present

day knowledge, he did restrict the family to include only Gram positive truly spherical cocci - the genus Streptococcus was at last excluded from the family. From 1933 until the present day the family Micrococcaceae has changed little in definition, the only exceptions being in the classifications of Kluyver & van Niel (1936), Prevot (1940, 1948), Tesic (1957) and Krassilnikov (1959), who have reverted to the pre-1933 conception of the family and include the genera Streptococcus and Neisseria. Even the number and names of genera of cocci within the Micrococcaceae have altered considerably in the last two decades, thus adding to the already existing confusion of the taxonomy of the family. Table 1 shows the generic names of Gram positive, truly spherical cocci and also the number of species, minor groups and subgroups within each genus as used by various taxonomists in the last 25 years. It can be seen from the table that whereas contributors to Bergey's Manual (6th Edition 1948, 7th Edition 1957) classified strictly aerobic, facultatively aerobic and strictly anaerobic cocci within the family Micrococcaceae, none of the more recent taxonomic studies of strains include the anaerobic cocci within this family. Indeed, the strictly anaerobic cocci are the least classified of all the members of the Micrococcaceae and require, even more than the strictly aerobic and facultatively aerobic strains, exhaustive taxonomic studies. The strictly aerobic and facultatively aerobic members of the family Micrococcaceae have not been fully

classified in recent years, despite many attempts to do so. Most of the classification schemes published in the last sixteen years have involved only a small number of strains, e.g. the 49 of Hill (1959) and the 37 of Rosypal et al. (1966), or have made use of a limited number of characters, e.g. the four characters of Shaw et al. (1951), or have been devised from strains isolated mainly from one habitat, e.g. fermented meat - Pohja (1960), human mouth - Pike (1962), sea water - Anderson (1962) and bacon - Baird-Parker (1963). Although contributors (Table 1) to Bergey's Manual (1957) classified this group of organisms thoroughly, their scheme is artificially constructed, being based on dichotomous keys of a few characters, and is much closer to an identification scheme than a classification scheme. Thus, there is a real need for an Adansonian classification scheme based on the observation of a large selection of characters exhibited by many aerobic and facultatively aerobic strains of the family Micrococcaceae, isolated from a variety of habitats.

Table 1

The genera into which Gram-positive, truly spherical cocci have been grouped by taxonomists in the last 25 years; numbers of species, subgroups, or minor groups in each genus are also listed

Authors	Genera						
	Micrococcus	Staphylococcus	Sarcina	Gaffkya	Methanococcus	Peptococcus	Undetermined group
6th edition of Bergey's Manual (1948); contributors - Hucker, 1943; Breed, 1943; & Hall, 1944	21+	-	9+	2+	-	-	609+*
Abd-el-Malek & Gibson (1948)	2	4	-	-	-	-	1
Shaw et al. (1951)	-	5	-	-	-	-	-
7th edition of Bergey's Manual (1957); contributors - Smit, 1949; Douglas, 1954; Breed, 1954, 1955; Barker, 1955; Hucker, 1954, 1955; & Evans, 1955	16	2	10+	2	2	11	-
Hill (1959)	1	2	-	-	-	-	2
Pohja (1960)	12	2	-	-	-	-	-
Anderson (1962)	17	-	-	-	-	-	-
Pike (1962)	2	4	1	1	-	-	-
Mossel (1962)	6	2	-	-	-	-	-
Baird-Parker (1963)	7	6	1	-	-	-	-
Baird-Parker (1965a)	8	6	-	-	-	-	-
Rosypal et al. (1966)	3	3	-	-	-	-	1

N.B. The genera *Methanococcus* and *Peptococcus* contain only strict anaerobes.

+ = includes species which are strict anaerobes.

* = an appendix of 609 named "species" of the following genera:- *Ascococcus*, *Aurococcus*, *Coccus*, *Galactococcus*, *Gynococcus*, *Jodococcus*, *Merismopedia*, *Methococcus*, *Micrococcus*, *Pediococcus*, *Pedioplana*, *Planococcus*, *Planosarcina*, *Rhodococcus*, *Staphylococcus*, *Tetracoccus*, *Urococcus* and *Urosarcina*.

GENERA WITHIN THE FAMILY MICROCOCCACEAE

The genera *Micrococcus* and *Staphylococcus*

There has been, and still is, confusion over the nomenclature and classification of micrococci and staphylococci. The main problem in nomenclature of these genera arose as early as 1872 when Cohn used the generic name *Micrococcus*, because he worked with a mixed culture, and unfortunately his type species *Micrococcus luteus* has so far not been re-isolated - that is to say that no organism has been found to fit his original description (Gibson, 1953; Evans *et al.*, 1955). The bacterial taxonomists of the late 19th and early 20th centuries, however, were not concerned with the technicalities of the naming of bacteria, since there was no code for the guidance of bacteriologists until 1917. Until the early 1940's taxonomists were less interested in distinguishing between the genera *Micrococcus* and *Staphylococcus* than they were in distinguishing the pathogenic from the saprophytic Gram positive cocci, and there was little attempt to look for differential criteria for the two genera or to establish their correct nomenclature. Between 1943 and 1959, however, there was considerable controversy regarding the dividing and naming of truly spherical cocci. The opinion of Abd-el-Malek & Gibson (1948) was that "the distinction between *Staphylococcus* and *Micrococcus* is at present quite indefinite", although their own work showed that the

Staphylococcus-Micrococcus complex appeared to be in a linear series with the pathogenic staphylococcus at one extreme and the thermoduric saprophyte at the other. In their contributions to the 6th edition of Bergey's Manual (1948), Hucker (1943) and Hall (1944) departed from the taxonomic position taken by all previous editions of Bergey's Manual (1923, 1925, 1930, 1934, 1939) that Staphylococcus was separable from Micrococcus, when they expressed the view, without giving a reason, that all Gram positive truly spherical cocci, excluding tetrad- and packet-formers, were all members of the genus Micrococcus. Shaw et al. (1951) differed little from Hucker (1943) and Hall's (1944) point of view as far as classification was concerned, when they considered that all aerobic, Gram positive, catalase positive cocci should be placed into one genus, but since Cohn's (1872) type species of Micrococcus, does not, and did not, exist, in the opinion of Shaw et al. (1951) the name Micrococcus is illegitimate. Instead they chose the name Staphylococcus for this one genus and, therefore, so far as nomenclature is concerned, Shaw et al. (1951) differ from all previous taxonomists studying this group of bacteria.

From a classification point of view, Van Eseltine (1955) was in agreement with the opinions of Hucker (1943), Hall (1944) and Shaw et al. (1951) when he came to the conclusion that there was insufficient evidence in the literature to support the

separation of the two genera, but he was less dogmatic about the naming of the genus "although *Micrococcus* Cohn 1872 clearly has priority and would appear to be the name of choice provided only that a suitable culture can be found to conform to the description of the type species". Elek (1959), however, completely agreed with Shaw *et al*. (1951) as far as both classification and nomenclature were concerned.

The uncertainties in the minds of taxonomists concerning the nomenclature and classification of micrococci and staphylococci were partly resolved by Evans *et al*. (1955) when they separated the two genera, *Micrococcus* and *Staphylococcus* on the basis of fermentation of glucose with production of acid under anaerobic conditions; those strains which did so he confined to the genus *Staphylococcus*, those that did not, or produced acid only in aerobic conditions, he put in the genus *Micrococcus*. Differentiation between the two genera by this one criterion - as far as classification and identification are concerned - has been accepted by Breed (1955) in the 7th edition of Bergey's Manual (1957), Mossel (1962), Baird-Parker (1963, 1965a) and the Subcommittee on Taxonomy of Staphylococci and Micrococci (1965b).

Although the work of Evans *et al*. (1955) was an important step forward in the taxonomy of the family *Micrococcaceae*, matrix diagrams of similarity coefficients in the Adansonian

classification schemes of Hill (1959), Pohja (1960) and Rosypal et al. (1966) do not indicate the existence of two clearly defined natural groups which correspond to the genera Micrococcus and Staphylococcus. In the words of Silvestri & Hill (1965a) "attempts to find a single character which, independently, can separate two groups such as staphylococci and micrococci are ... bound to fail". Thus it may be, that the state of flux in the taxonomy of the genera Micrococcus and Staphylococcus which has existed since the late 19th century will continue for some years to come.

The genus Sarcina Goodsir 1842

The genus Sarcina is recognised by some modern authorities (Smit, 1949, and Breed, 1955, in the 7th edition of Bergey's Manual, 1957; Krassilnikov, 1959; Pike, 1962; Baird-Parker, 1963), but other microbial taxonomists are of the opinion that the dividing line between the genus Micrococcus and the genus Sarcina is obscure (Abd-el-Malek & Gibson, 1948; Shaw et al. 1951; Kocur & Martinec, 1962; Baird-Parker, 1965a), and in fact Kocur & Martinec (1962) and Baird-Parker (1965a) considered that the name Micrococcus luteus is synonomous with the name Sarcina lutea. Although Breed (1943) and Smit (1949) in the 6th and 7th editions of Bergey's Manual respectively stated that the genus Sarcina contains both aerobic and anaerobic species, Shaw et al. (1951) and Cowan (1962) were of the opinion that

the name Sarcina should be restricted to anaerobic, packet-forming cocci, since the species on which the genus was founded, type species Sarcina ventriculi Goodsir 1842, is a strict anaerobe. Since there has been no Adansonian classification of aerobic and anaerobic species of Sarcina, it is not possible to know how many natural groupings occur in this genus. Obviously more taxonomic studies are necessary before any definitive position can be taken on the taxonomy of the genus Sarcina.

The genus Gaffkya Trevisan 1885

Gaffkya was reinstated as a genus by Prévot (1933) to contain Gram positive cocci occurring in clusters of tetrads lying in one plane. This same genus was recognised by Bergey (1934) in the 4th edition of Bergey's Manual, Kluyver & van Niel (1936), Bergey (1937) and Hucker (1943) in the 5th and 6th editions of Bergey's Manual respectively, Prévot (1948), Breed (1955), in the 7th edition of Bergey's Manual, and Pike in 1962. The opinion of taxonomists, on the whole, in recent years, however, is that the genus Gaffkya should be abandoned; Abd-el-Malek & Gibson (1948), Shaw et al. (1951), Mossel (1962), Baird-Parker (1963, 1965a) and Rosypal et al. (1966) do not use the generic name at all. It is worth noting, however, that taxonomists who do not accept the generic name Gaffkya, have not, in fact, examined strains from

the human mouth, and the only species of *Gaffkya* likely to be classified by these taxonomists is *Gaffkya tetragena*, which is found solely in the human respiratory tracts (the other species are rarely encountered, and are found in little studied habitats, i.e. *Gaffkya homari* occurs in diseased lobsters, and *Gaffkya anaerobia* occurs in the large intestine of the horse and human female genital tract). It is unlikely, therefore, that any of these taxonomists studied strains which were tetrad formers, and for this reason they would not be justified in excluding the genus from the family *Micrococcaceae*. Nevertheless, Pike (1962), who certainly examined strains of Gram positive, catalase positive cocci from the human mouth, used few characters to separate *Gaffkya* from other genera (Table 4).

In conclusion, therefore, there seems to be little evidence of the existence of a natural group in the family *Micrococcaceae*, a group that could be given generic status based on the definitions of Breed (1955) and Pike (1962) and named *Gaffkya*, but there is clearly scope for an Adansonian classification of Gram positive, catalase positive cocci from human and animal mouths, which would settle, once and for all, the question of the existence of the genus, *Gaffkya*, within the *Micrococcaceae*.

The genus *Sporosarcina* Orla-Jensen 1909

The interesting nature of *Sarcina ureae* (Beijerinck) Löhnis, the only representative of the cocci that is motile and produces endospores, was noted by Orla-Jensen (1909), and he placed the species in a new genus, *Sporosarcina*. The generic name *Sporosarcina* was only used once again in a classification scheme, that of Kluyver & van Niel (1936). All other taxonomists have, until recently, considered the species to be an anomalous member of the genus *Sarcina*, in the family *Micrococcaceae*. MacDonald (1962), Kocur & Martinec (1965b) and Mazanec *et al.* (1965), on the other hand, rejected it from the *Micrococcaceae* on the evidence of morphological, physiological and cell composition studies. These taxonomists considered that the spore structure and ellipsoidal nature of the cells excluded them from being truly spherical cocci and the above-mentioned authors decided that the genus *Sporosarcina* was more closely related to the family *Bacillaceae* than to the family *Micrococcaceae*. It seems, therefore, that *Sporosarcina ureae* is not a member of the family *Micrococcaceae*.

The anaerobic genera

The 7th edition of Bergey's Manual (1957) presents classification schemes of the little studied anaerobic genera *Methanococcus* (Barker, 1955) and *Peptococcus* (Douglas, 1954), but no taxonomic studies of these groups of organisms have been carried out since then; nor has any taxonomic

attention been paid to the anaerobic species of the genus *Sarcina*, since their classification in the same edition of Bergey's Manual (1957) by Smit (1949). Clearly the only authoritative classification so far of strictly anaerobic species within the family *Micrococcaceae* is to be found in the 7th edition of Bergey's Manual (1957).

The species of the genera *Micrococcus* and *Staphylococcus*

There are many problems concerned with the nomenclature and identification of *Micrococcus* and *Staphylococcus* species, but these problems have arisen because no standard classification scheme of these species has been universally accepted. The following section of the review of the literature dealing with the taxonomy of the species of *Micrococcus* and *Staphylococcus* shows the lack of agreement among taxonomists in defining these species, and this has led in turn to the considerable confusion of nomenclature and identification of these species in the literature.

The species of the genus *Micrococcus*

Micrococcus luteus (Schroeter 1872) Cohn 1872

Although *Micrococcus luteus* is not now considered the type species of the genus by some authorities, e.g. Shaw *et al.* (1951), other taxonomists accept this organism to be the true type species (Evans *et al.*, 1955; Hucker 1954, in the 7th edition of Bergey's Manual, 1957; Kocur & Martinec 1962). Hucker (1954), Kocur & Martinec (1962) and Baird-Parker (1965a)

agreed that Micrococcus luteus contains biochemically inactive strains, which produce a yellow pigment, but Hucker (1954) defined his species as being able to produce acid from glucose, sucrose and mannitol, whereas Kocur & Martinec (1962) considered that strains of this species did not utilise any mono- or di-saccharides. In fact, manometric studies on strains of Micrococcus luteus by Nunheimer & Fabian (1942) and Rosypal & Kocur (1963) have shown that this species is capable of utilising a variety of carbon compounds, including the "sugars," glucose, sucrose and maltose, but it seems that little acid is produced and is not easily detected in a complex medium. Baird-Parker (1965a) came to the conclusion that Micrococcus luteus was weakly glucose-oxidising, or unable to utilise glucose at all.

Micrococcus roseus Flugge 1886

The name Micrococcus roseus appears to have universal acceptance at the present time, except that Shaw et al. (1951) referred it to the genus Staphylococcus, for reasons given on page 13, but definitions of this species vary considerably. Shaw et al. (1951) considered the essential characters to be, coagulase not produced, acid but no acetoin produced from glucose and pink colonies on agar media. Hucker (1954) in Bergey's Manual (1957) did not mention that glucose is utilised but he stated that apparently glycerol and mannitol are. If glycerol and mannitol are utilised, however, then

glucose must be also, unless strains of Micrococcus roseus possess a hitherto undiscovered and unique carbohydrate metabolic pathway. Kocur & Martinec (1962) stated that growth of Micrococcus roseus strains in peptone water containing carbohydrates produced no acid from 17 carbohydrates tested, including glucose, glycerol and mannitol. Baird-Parker (1965a) came to the conclusion that whereas some strains of Micrococcus roseus may be able to oxidise glucose weakly, the detection of small amounts of acid released into a complex medium is not always possible by observing the change of colour of a pH indicator.

Other Micrococcus species

All other specific micrococcal names are not universally accepted at present. The number of species listed within the genus Micrococcus has varied greatly in recent classification schemes; the numbers range from the preponderance of 609 named "species" collated by Hucker (1943) and Hall (1944) in the 6th edition of Bergey's Manual (1948), and of 60 species classified by Krassilnikov (1959), to the modest selection of 6 species of Kocur & Martinec (1962).

Micrococcal strains, however, have been classified into groups or subgroups, rather than into species, by other taxonomists in recent years, e.g. Pohja (1960), Pike (1962), Baird-Parker (1963) and Rosypal et al. (1966), and these are

listed in Table 1.

The species of the genus *Staphylococcus*

Staphylococcus aureus Rosenbach 1884

Controversy has arisen around the naming of the type species of the genus *Staphylococcus*. Breed (1956) and Elek (1959) were of the opinion that the name originally given by Rosenbach (1884) to the type species was *Staphylococcus pyogenes*, but Cowan (1955, 1956) maintained that *Staphylococcus aureus* was the first correct binomial name, used by Rosenbach (1884) to name orange pus-forming staphylococci, the type species of the genus. In fact, the latter name was decided upon by the Judical Commission of the International Committee for Bacterial Nomenclature (1958) for the type species of the genus *Staphylococcus*. As far as classification and identification are concerned, all strains of *Staphylococcus* which produce coagulase are generally recognised as belonging to the species *Staphylococcus aureus*.

In recent years when some taxonomists have preferred to give numbers or letters rather than generic and specific names to their classified groups or subgroups of strains of micrococci and staphylococci, the definition of one group or subgroup always corresponds to that of *Staphylococcus aureus*, e.g. subgroup Ia of Pike (1962), subgroup I of Baird-Parker (1963, 1965a), group 7 of Rosypal *et al.* (1966); (Tables 1,

Table 2

Classification scheme of Abd-el-Malek & Gibson (1948)

*Differential characters of the staphylococci and micrococci of milk**

	I Staphylococcus group				II Intermediate group	III Dairy micrococci	
	A	*B*	*C*	*D*		*A*	*B*
Acid from glucose	+	+	+	+	–	+	+
NH_3 from arginine	+	+	+	–	–	–	–
Acetoin formation	+	+	– or weak	– or weak	–	–	–
Coagulase production	+	–	–	–	–	–	–
Acid from mannitol	+	–	V	V	–	–	–
Survival in milk at 60° C. 30 min.†	–	–	–	–	V	+	+
Acid from glycerol	V	V	V	V	–	–	+
Pigment on agar	Yellow, orange, fawn or none				Greenish yellow or none	Greenish yellow	Pale greenish yellow or none
Principle source	Animal body					Dairy equipment	

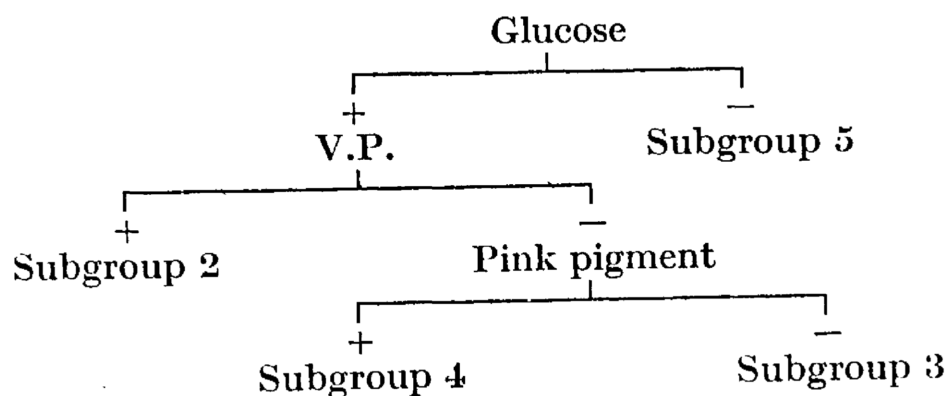
V = variable.

* Uncommon exceptions are mentioned in the text.

† Strains showing slight survival, as indicated by the occasional production of a small number of colonies (< 10) when the heated suspension is plated, are regarded as being killed (–).

Table 3

Classification scheme of Shaw *et al* (1951) for coagulase negative staphylococci



Subgroup 1 = *Staphylococcus aureus* (coagulase positive strains)

Subgroup 2 = *S. saprophyticus*

Subgroup 3 = *S. lactis*

Subgroup 4 = *S. roseus*

Subgroup 5 = *S. afermentans*

Table 4

Classification scheme of Pike (1962)

Typical characters of group I (Staphylococcus) *strains and a comparison of this group with the remaining groups*

Test	No. of strains and reactions* of groups							
	Ia (40)	Ib (102)	Ic (57)	Id (63)	II (30)	IIIa (7)	IIIb (6)	IV (31)
Coagulase†	+	−	−	−	−	−	−	−
Mannitol, anaerobic†	+	−	−	−	−	−	−	−
Phosphatase†	+	±	−	−	−	−	−	−
Haemolysis†	+	+	−	−	−	−	−	−
Egg yolk reaction†	+	+	−	−	−	±	−	−
Acetoin†	+	+	+	−	+	±	−	−
Glucose, anaerobic†	+	+	+	−	±	−	−	−
Gold-white smooth colony‡	+	+	+	+	−	−	−	−
15% NaCl broth‡	+	+	+	+	−	−	−	−
'Clump' morphology‡	+	+	+	+	−	−	±	−
Hippurate‡	+	+	+	+	−	±	−	−
Tributyrin‡	+	+	+	+	−	±	±	+
Glucose, aerobic‡	+	+	+	+	+	+	±	−
Optimum temp. 28°–37°‡	+	+	+	+	+	+	−	+

* Reactions: +, displayed by 76–100% of strains; ±, 26–75%; −, 0–25%.
† Distinguishing characters of group I strains. ‡ General characters of group I strains.

Typical characters of groups II (G. tetragena), *III* (*miscellaneous* Micrococcus *spp.*) *and IV* (Sarc. lutea), *and a comparison of these groups with group I*

Test	No. of strains and reactions* of groups							
	Ia (40)	Ib (102)	Ic (57)	Id (63)	II (30)	IIIa (7)	IIIb (6)	IV (31)
Colonies white, conical	−	−	−	−	+	−	−	−
'Tetrad' morphology	−	−	−	−	+	+	±	−
Acetoin	+	+	+	−	+	±	±	−
Nitrate reduction	+	+	±	±	+	+	±	−
Glucose, aerobic	+	+	+	+	+	+	±	−
Optimum temp. 20°–28°	−	−	−	−	−	−	+	−
Tributyrin	+	+	+	+	−	±	±	+
5% NaCl broth	+	+	+	+	−	+	+	+
10% NaCl broth	+	+	+	+	−	+	±	+
Starch	−	−	−	−	−	+	±	+
Casein	±	±	−	−	−	−	±	+
'Packet' morphology	−	−	−	−	−	−	−	+

Table 5

Classification scheme of Baird-Parker (1963, 1965a)

Main characters of subgroups within Group 1 (Staphylococcus *Rosenbach emend. Evans*)

Characteristic	Subgroups I	II	III	IV	V	VI
Coagulase	+	−	−	−	−	−
Phosphatase	+	+	+	−	−	−
Mannitol (aerobic)	+ (usually)	−	−	−	−	+
Mannitol (anaerobic)	+ (usually)	−	−	−	−	− (usually)
Acetoin	+	+	−	+	+	+
Lactose	+ (usually)	+ (usually)	v	−	+	v
Maltose	+	+	−	v	+	v
Growth at 10°	+ (usually)	−	−	−	−	− (usually)
No. of isolates	24	854	24	53	19	96

v = variable

Main characters of subgroups within Group 2 (Micrococcus *Cohn emend. Evans*)

Characteristic	Subgroups 1	2	3	4	5	6	7
Acid from glucose (aerobic)	+	+	+	+	+	+	−
Phosphatase	−	−	−	−	−	+	−
Acetoin	+	+	+	+	−	−	−
Average end pH value in 2% w/v glucose broth	4·6	5·1	5·0	5·2	5·5	5·3	6·5
Arabinose	−	−	−	+	v	+	−
Lactose	−	+	v	+	+ (usually)	+	−
Maltose	v	+	+ (usually)	+	+ (usually)	+	−
Mannitol	−	−	+	+	+ (usually)	+	−
Lipolysis	v	+	+	−	− (usually)	+ (usually)	v
Tween hydrolysis	− (usually)	−	v	−	−	+	v
Growth at 10°	− (usually)	+ (usually)	+ (usually)	+	+ (usually)	+	+ (usually)
No. of isolates	17	8	75	10	544	4	10

v = variable

Strains which have similar characteristics to those in Micrococcus subgroup 7, but are red pigmented, are placed in Micrococcus subgroup 8.

2, 3, 4, 5, 12 and 13).

Other *Staphylococcus* species

Other specific epithets of the genus *Staphylococcus* are often used without reference to the original definitions, which are given below, e.g. Loeb *et al.* (1950), Jacobs *et al.* (1964).

Staphylococcus albus - staphylococci other than *Staphyloccus aureus*, the two species being distinguished only by pigment: Rosenbach (1884).

Staphylococcus citreus - lemon pigmented cocci; Passet (1885).

Staphylococcus epidermidis - white pigmented cocci; Winslow & Winslow (1908).

Staphylococcus saprophyticus - coagulase negative strains of human and animal origin; Fairbrother (1940). The species was re-defined by Shaw *et al.* (1951) to include coagulase negative strains, which produce acid and acetoin from glucose.

Staphylococcus lactis - coagulase negative strains which produce acid but not acetoin from glucose and whose colonies are not pink; Shaw *et al.* (1951).

Staphylococcus afermentans - coagulase negative strains which do not produce acid or acetoin from glucose; Shaw *et al.* (1951).

Comments on the definitions of specific names of the genus *Staphylococcus* by subsequent taxonomists

Evans (1955), in his contribution to the 7th edition of Bergey's Manual (1957), included only two species in the genus

Staphylococcus - aureus and epidermidis, and he defined the latter species as coagulase negative, glucose but not mannitol fermentative. Hill (1959) noticed a resemblance between Staphylococcus epidermidis, as re-defined by Evans (1955), and a group of 3 strains, which he called Staphylococcus saprophyticus, according to the definition of Shaw et al., 1951 (page 93). Jones et al. (1963) also noticed this resemblance and they considered that the two specific names wer synonomous. In my opinion, however, they could hardly be described as such for the following reasons:- Hill's (1959) description of his 3 Staphylococcus saprophyticus strains is "mannitol often positive", when incubation was carried out under aerobic conditions, but true fermentation was not investigated. According to Evans (1955), Staphylococcus epidermidis produces acid from mannitol neither under aerobic nor anaerobic conditions. If aerobic or anaerobic production of acid from mannitol is an important taxonomic criterion, it follows that the two species cannot be the same. Baird-Parker (1963) also noted this discrepancy and his own taxonomic work showed that the 2 species were not the same.

In 1963 Jones et al. expanded Evans' (1955) definition of Staphylococcus epidermidis to be the ability of the species to grow anaerobically in a defined complex medium with glucose or pyruvate as the energy source, the ability to ferment serine

as an energy source, the requirement for biotin and uracil as vitamins under anaerobic conditions in a semi-synthetic medium, and the inability to reduce nitrate to nitrite or to produce coagulase. Using different morphological and physiological characters as taxonomic criteria, Pike (1962), Baird-Parker (1963, 1965a) and Rosypal *et al*. (1966) have divided strains of staphylococci into groups and subgroups (Tables 4, 5 and 20). Pike (1962) and Baird-Parker (1963) could not correlate their subgroups of coagulase negative staphylococci with the definition given by Evans (1955) of *Staphylococcus epidermidis* or the definitions given by Shaw *et al*. (1951) of *Staphylococcus saprophyticus*, *Staphylococcus lactis* and *Staphylococcus afermentans*. (page 23).

CHARACTERS USED IN TAXONOMIC STUDIES OF MICROCOCCI AND STAPHYLOCOCCI

In the following section, the characters that have been, and are used for classifying members of the family *Micrococcaceae*, in particular micrococci and staphylococci, are reviewed. The term "character" is taken to be a property exhibited by a bacterial strain; the character may be the property of the morphology of the cells, e.g. cell shape or colonial size, or it may be the property of the physiology of the cells, e.g. pigment production or hydrolysis of casein, or it may be the property of the chemical structure of parts of bacterial cells, e.g. cell wall or DNA base composition characters.

There are 3 questions concerning this section that the reader is likely to ask:-

(1) Which characters have been used for classifying micrococci and staphylococci?

(2) Different taxonomists, examining the same characters exhibited by micrococci and staphylococci, often obtain conflicting results; why should there be such discrepancies?

(3) Have the taxonomists used these characters in an unbiased way for classifying these bacteria?

1. The use of characters

The earlier taxonomists used what information was available about the bacteria, i.e. their morphology and simple physiology,

as characters for taxonomic studies. As the knowledge of bacteria increased, through advances in chemical and biochemical techniques, more information became available for taxonomic purposes, e.g. cell wall analysis characters. Consequently, at present, there are many characters which can be used for classifying micrococci and staphylococci.

Very few taxonomists, however, have compared classification schemes based on morphological and physiological characters, with those schemes, for instance, based on DNA base composition characters, and therefore, in this review it has been difficult to compare the results of one taxonomist with the results of another. In fact, no taxonomist has, so far, used all known characters possessed by these bacteria in a classification scheme.

2. Discrepancies between results of different taxonomists examining the same characters

This is a more difficult question to answer. There are, however, probably three main reasons for these discrepancies, (a) taxonomists throughout the years have used strains normally isolated by themselves for their studies, and the classification of the same named culture collection strains by different taxonomic methods and different taxonomists is comparatively new. The sources of the strains are bound to influence the properties of the strains, and so micrococcal and staphylococcal strains isolated, for instance, from milk

or milk products are likely to have different characteristics from strains isolated from human or animal skin. All taxonomists should use at least some of their own isolates in their classification schemes, otherwise new species or subgroups etc. would never be discovered, but not to the exclusion of culture collection strains.

(b) discrepancies in results also may be due to the variations in experimental conditions, e.g. variation in the length of time of incubation, temperature of incubation, composition of media, and sensitivity of test reagents.

(c) the confusion in the nomendature of these organisms over the last 20 years must contribute largely to the discrepancies in the results. For without a universally recognised system of nomenclature the taxonomist must define the organisms he names; this in many cases he has failed to do. For instance, some authors refer to "staphylococci" without defining them - it is possible that some of these organisms are micrococci, i.e. they do not ferment glucose.

My inability, however, to answer this question completely has led me to repeat, with my own strains, many of the tests used by previous taxonomists for characterising these bacteria.

3. <u>The use of characters for classifying in an unbiased way</u>

The answer to this question must in many cases be "no", although to confirm my opinion, I have had to critically examine many of the characters, which have been used as criteria

for classifying micrococci and staphylococci, and I shall discuss the significance of the use of main characters in classification schemes later in this thesis (page 157).

Most of this section is devoted to a discussion of the characters used for the classification of micrococci and staphylococci, but after the discussion of the physiological characters there is a brief comparison of the main morphological and physiological classification schemes produced in the last 20 years; these schemes are the ones most widely used by bacteriologists for classifying micrococci and staphylococci, and knowledge of these schemes is an essential background in reading this thesis.

MORPHOLOGICAL CHARACTERS

Shape of the Cells

By definition all micrococci and staphylococci are spherical in shape (Migula, 1900; Breed, 1955, in Bergey's Manual, 7th edition, 1957). However, differentiation between a slightly ellipsoidal coccus and a very short rod is by no means easy; 7 National Collection of Type Cultures (N.C.T.C.) strains of *Micrococcus* and *Staphylococcus* species were rejected from a scheme of classification by Shaw *et al*. (1951) on the grounds that, under certain conditions, these organisms developed into branching rods or became definitely ellipsoidal in shape. Baird-Parker (1963) found that some of the Gram positive, catalase positive cocci he studied were slightly ellipsoidal in shape, but he did not reject them from his classification scheme. It seems from the literature that if cells of these bacteria possess a slight degree of ellipticity and not a cocco-bacillus shape, they can be classified with the truly spherical cocci.

Size of the Cells

Great variation has been found in the size of cells within the family *Micrococcaceae* as Table 6 shows. Abd-el-Malek & Gibson (1948), Pike (1962) and Baird-Parker (1963) considered that the cells of micrococci giving weak biochemical reactions are larger than the cells of fermentative staphylococci. Hill (1959) observed that his micrococci were pleomorphic, but

Table 6

Sizes of cells within the family *Micrococcaceae* as found by various authors

Authors	Diameter of cells in μ
Abd-el-Malek & Gibson (1948)	0.7 to larger sizes
Shaw *et al.* (1951)	0.75 to 2.0
Hill (1959)	0.6 to 1.3
Anderson (1962)	0.5 to 3.0
Pike (1962)	0.7 to 1.2 & larger cocci
Kocur & Martinec (1962)	1.0 to 1.6 (micrococci only)
Baird-Parker (1963)	Less than 1.0 to 1.5

Anderson (1962) noticed that his fermentative organisms were more irregular in shape than his non-fermentative ones.

Motility of the Cells

Motile strains of truly spherical cocci are rarely encountered. Contributors (Table 1) to the 6th edition of Bergey's Manual (1948) stated that 5 of 34 species within the family *Micrococcaceae* were motile, and contributors (Table 1) to the 7th edition of Bergey's Manual (1957) found that 5 of their 43 species were also motile. Shaw *et al*. (1951) found that only 3 of 432 strains were motile, and the motile strains possessed 1 flagellum per cell. Baird-Parker (1963), however, could find no motile strains in the 1,250 strains he studied.

Colonial Form of the Cells

Taxonomists generally agree that colonial morphology is of limited use for classifying micrococci and staphylococci, and consequently there is little reference to this character in taxonomic publications. Shaw *et al*. (1951) and Baird-Parker (1963), however, recorded their observations. Shaw *et al*. (1951) noted that their colonies were low, round, convex, smooth and entire, although some were rough and some were crenulate. Baird-Parker (1963), considered most of his colonies to be 0.5-2 mm. in diameter, convex, and usually smooth, although some were crenulate. Any large granular, matt, colonies were composed of packet-forming cocci.

Appearance of Cells in Broth Culture

Shaw *et al.* (1951) and Baird-Parker (1963) found that staphylococci usually grow as an easily suspendible fine sediment in broth culture, but that other strains (some micrococci, according to Baird-Parker, 1963) produced a more mucoid or granular deposit, which was difficult to disperse in the broth. Although Shaw *et al.* (1951) and Baird-Parker (1963) agreed in their findings on the growth of Gram positive, catalase positive cocci in broth, they did not place any taxonomic significance on their observations.

Gram's Reaction of Cells in Stained Smears

The confusion of the early taxonomists concerning the Gram's reaction of cells of micrococci and staphylococci has been reviewed on page 8. In 1965, Baird-Parker stated that all micrococci and staphylococci are defined as being Gram positive, and consequently he considered that several of the established *Micrococcus* species were not true members of the family *Micrococcaceae*. Cell wall studies of 2 Gram negative *Micrococcus* species - *M. denitrificans* (Baird-Parker, unpublished work) and *M. halodenitrificans* (Smithies *et al.*, 1955), and of 1 Gram positive species *M. radiodurans* (Work, 1964), have shown that these species possess characteristic Gram negative cell wall components. These species, according to Baird-Parker (1965a), should be re-classified with the Gram negative genera.

The Gram reaction, therefore, is one of the first characters that a taxonomist should examine, when he is trying to classify micrococci and staphylococci and it is interesting to find that Abd-el-Malek & Gibson (1948), Pohja (1960), Mossel (1962) and Pike (1962) did not mention the Gram's reaction of the strains they classified; it is probable that these authors assumed that all the strains they examined were Gram positive.

PHYSIOLOGICAL CHARACTERS

The Optimum Temperature for Incubation of Cultures

The temperatures for the incubation of cultures of micrococci and staphylococci are sometimes arbitrarily chosen, as when the Subcommittee on Nomenclature and Taxonomy of Staphylococci and Micrococci (1965b) chose 37°C, Baird-Parker (1963) 30°C, Evans *et al.* (1955) and Anderson (1962) 20°C, although the last author was working with sea water micrococci, which have low optimum temperatures for growth (18°C - 22°C ZoBell, 1946). Shaw *et al.* (1951), Hill (1959), Pohja (1960) and Pike (1962), however, found the optimum temperature of growth of their strains before embarking on a series of critical experiments, while engaged on a study of the taxonomy of micrococci and staphylococci. Shaw *et al.* (1951) found that all of their 432 strains grew at 22°C and most strains had their optimum temperature at 30°C or 37°C.

The Use of Temperature Growth Range in Classification

Hucker (1954), Kocur & Martinec (1962), Jones *et al*. (1962) and Baird-Parker (1963, 1965a) are the only taxonomists to use temperature growth range as a criterion in classification. Hucker (1954; 7th edition of Bergey's Manual, 1957) and Kocur & Martinec (1962) defined a new species of *Micrococcus* (named *cryophilus*) on the basis of its low temperature range - 4°C to 23°C, with the optimum at 10°C, and thus demonstrated the use of temperature growth range as a main taxonomic criterion.

Jones *et al*. (1962) and Baird-Parker (1963) used growth at 10°C and 45°C as characters in their respective classifications. Their results are recorded in Table 7. Jones *et al*. (1962) made no comment on the usefulness of growth at either temperature, but Baird-Parker (1963) recorded that "growth at 45°C was a more variable character" than growth at 10°C; Baird-Parker (1963) used growth at 10°C as a main character in his classification scheme (Table 5).

Resistance of Organisms to Heat

Abd-el-Malek & Gibson (1948) were the first taxonomists to characterise a group of micrococci and staphylococci on the basis of resistance to heat. Their *Staphylococcus* group I (Table 2) strains failed to survive in milk heated at 60°C for 30 minutes, while strains of their Dairy Micrococci group

Table 7

The growth of strains of micrococci, staphylococci and sarcinas at 10°C and 45°C

Authors	Strains examined	Strains growing at	
		10°C	45°C
Jones *et al.* (1963)	15 strains of *Staphylococcus epidermidis*	6.7%	60%
Baird-Parker (1963) Classification Scheme - Table 5	24 strains of coagulase +ve staphylococci	80%	100%
	371 strains of coagulase -ve staphylococci	5%	92%
	352 strains of glucose oxidising micrococci	97%	11%
	9 strains of micrococci unable to utilise glucose	78%	78%
	2 strains of sarcina	100%	0%

III (Table 2) were resistant to such treatment. This interesting finding has not yet been confirmed by any other taxonomist. Hill (1959) also used heat sensitivity as a taxonomic criterion by heating cultures at 56°C and examining them after 5, 10, 20 and 30 minutes for viable organisms, but he did not state his results.

Sodium Chloride Resistance

Since many Gram positive, catalase positive cocci can be isolated from an environment with a high salt concentration, it was natural for some authors to examine the effect of sodium chloride on the growth of these organisms. In recent years, Hill (1959), Pohja (1960), Pike (1962) and Baird-Parker (1963) developed distinguishing tests, based on sodium chloride resistance, for the classification of strains into groups. Experimental details and results are given in Table 8. Pohja (1960), who is the only taxonomist to use sodium chloride resistance as a main character, arranged his strains into 3 groups; he used the term "halotolerant" for strains that grew on concentrations of sodium chloride up to 15%, and "non-halotolerant" for strains that did not grow on concentrations as high as 15% sodium chloride. Strains which grew on 15% sodium chloride agar, but not on 0% sodium chloride agar were given the name "halophilic".

Table 8

The effect of various concentrations of sodium chloride on the growth of Gram positive catalase positive cocci

Authors	Medium containing NaCl	No. of strains examined	NaCl concns. examined	Results
Pohja (1960)	Meat extract glucose agar	171	0%, 5% 10%, 15%	97% strains grew on 0% NaCl 99% strains grew on 5% NaCl 96% strains grew on 10% NaCl 86% strains grew on 15% NaCl
Hill (1959)	Nutrient agar	49	9%, 12% 15%	results not given
Pike (1962) Classification Scheme - Table 4	Nutrient broth	336	5%, 10% 15%	76-100% of strains in all Pike's (1962) groups grew in 5 & 10% NaCl, except for 30 *Gaffkya* strains (group II), none of which grew in any % NaCl tested, and for group IIIb of which only 26-75% of strains grew in 10% NaCl Only strains in group I grew in 15% NaCl
Baird-Parker (1963) Classification Scheme - Table 5	Yeast extract glucose broth	1250	0%, 5% 15%	99.7% strains grew on 0% NaCl 100% strains grew on 5% NaCl 86.6% strains grew on 15% NaCl

Prototroph Activity - Growth in Glucose Ammonium Phosphate Medium

Hucker, in his classification monographs (1924a,b) and in his contributions to the 6th (1948) and 7th (1957) editions of Bergey's Manual emphasises the character of prototroph activity, by the ability or inability of micrococci and staphylococci to grow in glucose ammonium phosphate medium. Shaw *et al.* (1951) and Evans *et al.* (1955) stated that growth on a minimal medium is dependent upon the carrying over of required vitamins, and this is probably the reason that the prototroph results of Shaw *et al.* (1951) were found to be variable. Many taxonomists have used glucose ammonium phosphate medium but have not considered growth in it to be an important criterion in their taxonomic studies (Thatcher & Simon, 1957; Pohja, 1960; Anderson, 1962; Baird-Parker, 1963).

The contributors (Table 1) to both editions of Bergey's Manual (1948, 1957) recorded that 30 - 31% of the species in the family *Micrococcaceae* were prototrophic, and Pohja (1960) found that 35% of his 171 strains were able to grow in glucose ammonium phosphate medium. Baird-Parker (1963), however, noted that only 19% of his 1,250 strains grew on this prototroph medium. It seems, from the results of these authors, that the majority of organisms able to grow on glucose ammonium phosphate medium are micrococci with weak, or no, biochemical activity.

Pigmentation

Many strains of cocci produce striking yellow, orange, golden and red pigments. This easily-observable fact probably led the early bacterial taxonomists to put great emphasis on the character of pigmentation for their classification schemes (Cohn, 1872; Winslow & Rogers, 1906; Winslow & Winslow, 1908; Hucker, 1924a,b). Later research work, however, is critical of the assumption that pigmentation is a consistent character and can be relied upon as a satisfactory taxonomic criterion. (Rahn, 1929; Abd-el-Malek & Gibson, 1948; Van Eseltine, 1955).

Pigment production by bacteria is dependent upon the composition of the media used and the incubating conditions, e.g. temperature and light; Table 9 shows the media and incubating conditions for pigment production of micrococci and staphylococci used by various authors in recent years.

Assessment of colour

Since assessment of colour is subjective, comparison of pigment with a system of colour charts was proposed and used as long ago as 1908 by Winslow & Winslow. This system was recommended by Lenning (1954) and Paclt (1958), and used once more, in a classification of marine micrococci by Anderson (1962). It seems strange, nevertheless, that Anderson (1962) does not mention the colour of any pigment, after he had taken

Table 9

Demonstration of pigmentation by Gram positive catalase positive cocci as recorded by authors in recent years

Authors	Medium	Incubating conditions	Pigments
Hucker (1943) from 6th edition of Bergey's Manual (1948)	Potato	Not mentioned	White Yellow Orange Pink, Red
Abd-el-Malek & Gibson (1948) Classification Scheme - Table 2	Meat extract agar, potato, 20-30% milk agar.	Not mentioned	Yellow, Greenish Yellow, Orange, Fawn
Shaw *et al.* (1951) Classification Scheme - Table 3	Nutrient agar potato	Optimum temperature for 24hrs & then kept for a week at room temperature in diffused light	Gold Lemon Pink
Hill (1959)	30% milk agar	37°C for 24 hrs. and then kept for 3 days at room temperature	White Gold Yellow Pink
Pohja (1960)	Glucose meat extract yeast agar containing various quantities of NaCl	25°C for 7 days	Grey White Yellow
Anderson (1962)	10% cream in a basal medium for marine micrococci	Room temperature for 14 days in diffused light	Colour not mentioned
Pike (1962) Classification Scheme - Table 4	33% skim milk agar (Christie & Keogh 1940)	Optimum temperature for 2 days & then kept for 5 days at room temperature in diffused light	White Gold Yellow Buff
Kocur & Martinec (1962)	Glucose yeast extract agar	30°C for 4-6days	Violet, Yellow Orange, Pink Yellowish Green, White
Mossel (1962)	Mannitol salt phenol red agar	37°C for 48 hrs.	White, Gold

Table 9 (continued)

Authors	Medium	Incubating conditions	Pigments
Willis & Turner (1962)	Glycerol monoacetate agar	37°C in the dark for 48 hours	White Yellow Orange Buff
Baird-Parker (1963) Classification Scheme - Table 5	Yeast tryptone agar containing 20% skim milk	30°C for 7 days	White Orange Yellow
Rosypal *et al.* (1966) Classification Scheme - Table 20	Glucose yeast extract agar	30°C for 4-6 days	Yellow Violet Pink

the trouble to use a colour chart. Most authors, however, confine their characterisation to a few principal colours, and these can be seen listed in Table 9.

Variation of pigment production

Abd-el-Malek & Gibson (1948), Shaw *et al.* (1951) and Baird-Parker (1963) agreed that pigmentation is not a stable character, since it is dependent on the conditions of growth of organisms. The variation of pigment production by *Staphylococcus aureus* has been known for some time. Andrewes & Gordon (1907), Jay (1962), Mossel (1962) and Baird-Parker (1963) showed that pigmented strains (yellow and orange) of micrococci and staphylococci often give rise to physiologically identical white variants.

Within strains of the family *Micrococcaceae*, Cowan (1962) and Kocur & Martinec (1962) recognised only 2 pigments - red and yellow - and Baird-Parker (1965a) only 1 pigment - red, to be stable; both these pigments were produced by strains of micrococci. Willis & Turner (1962), using a glycerol mono-acetate medium, claimed, however, that they could separate the saprophytic, coagulase negative staphylococci from the pathogenic, coagulase positive strains on the basis of pigmentation and the coagulase positive group could be further subdivided into three stable colour varieties - yellow, orange and buff.

Utilisation of Carbohydrates

The use of carbohydrates in early classification schemes

Andrewes & Gordon (1907) and Dudgeon (1908) were the first taxonomists to use the production of acid from carbohydrates by micrococcal and staphylococcal strains for classification purposes. From the materials available and the knowledge of bacterial biochemistry at that time, it is not surprising that Cummins & Cumming (1913) considered that the tests of the former authors were unreliable. In fact, their own results showed that the production of acid from carbohydrates was variable and they concluded such tests were difficult to use for subgrouping cocci.

Production of acid from glucose

All the recent attempts at classification have included acid production from glucose as an important criterion, e.g. Abd-el-Malek & Gibson (1948), Shaw et al. (1951), Hill (1959), Pohja (1960) and Baird-Parker (1963). Abd-el-Malek & Gibson (1948) and Shaw et al. (1951) failed to differentiate between fermentation and oxidation of "sugars" in their respective classification schemes, as had all previous taxonomists, and this was pointed out by Evans et al. (1955), who stated that ammonia produced from the nitrogenous constituents of the medium under aerobic conditions can mask acid production from glucose.

Evans *et al.* (1955) characterised strains in the genus *Staphylococcus* by their ability to grow and produce acid from glucose under anaerobic conditions, and strains of the genus *Micrococcus* by their inability to produce acid from glucose under anaerobic conditions. Thus strains belonging to either of the 2 genera could be separated by an oxidation-fermentation test.

The work of Richardson (1936), Gretler *et al.* (1955), Evans *et al.* (1955) and Jones *et al.* (1963) has shown that the vitamins uracil and biotin are necessary for the growth of staphylococci under anaerobic conditions, and thus a complex medium must be used to demonstrate the fermentation of glucose and other "sugars" by members of the family *Micrococcaceae*.

One of the main problems in testing bacteria for their fermentative ability is the maintainance of anaerobic conditions. Taxonomists have differed in their approach to achieve anaerobiosis, and Table 10 lists the media used for the demonstration of oxidation and fermentation of glucose and the methods for obtaining anaerobic conditions by various taxonomists.

Cowan & Steel (1964) compared the media and methods of different authors and found there was considerable variation in the results obtained with the same strains. The best agreement was obtained when anaerobic fermentation of glucose

Table 10

Utilisation of glucose by micrococci and staphylococci as demonstrated by various authors

Authors	Medium containing glucose	Conditions of incubation	Differention between oxidation and fermentation	Method of achieving anaerobic conditions
Abd-el-Malek & Gibson (1948)	Peptone water	7 days at optimum temp.	No.	-
Shaw et al. (1951)	Peptone water	14 days at 37°C or 21 days at 30°C or 28 days at 22°C	No.	-
Evans et al. (1955)	Tripticase yeast extract broth	10 days at 20°C	Yes	Vaspar cover on tubes
Thatcher & Simon (1957)	Tripticase yeast extract broth	10 days at 20°C	Yes	Vaspar cover on tubes and Brewer jars
Pohja (1960)	Casitone agar	10 days at optimum temp.	Yes	Vaspar cover on tubes
Mossel (1962)	Tripticase yeast extract agar (mannitol as sugar)	4 days at 36°C	Yes	Acid produced in lower half of agar deeps
Anderson (1962)	Sea-water basal agar medium with peptone and yeast extract	4 days at 20°C	Yes	Liquid paraffin cover
Pike (1962)	Peptone water	4 days at optimum temp.	Yes	Incubated under hydrogen
Baird-Parker (1963)	Mineral salt yeast extract agar	10 days at 30°C	Yes	Liquid paraffin cover
Cowan & Steel (1965; page 53)	Not mentioned	Not mentioned	Yes	Incubated in anaerobic jars
Subcommittee on taxonomy of micrococci and staphylococci (1965b)	Triptone yeast extract agar	5 days at 37°C	Yes	Paraffin oil cover

was tested by Baird-Parker's (1963) method, by Evans' (1955) method when a final pH of less than 5.0 was required, and by peptone waters containing the carbohydrates in an anaerobic atmosphere of hydrogen gas (see Table 10 for details of media and experimental conditions).

Many micrococci apparently do not break down glucose, even under aerobic conditions, and these are classified into a group by themselves, the characteristics of the group being based mainly on negative biochemical reactions - Staphylococcus afermentans (Shaw et al., 1951), "miscellaneous micrococci" (Hill, 1959), group IV or Sarcina lutea (Pike, 1962), and Micrococcus group 7 (Baird-Parker, 1963). In my opinion, strains which are biochemically inactive and have mainly negative reactions in common are not necessarily closely related, and should not be placed into a well-defined group as has been done by Shaw et al. (1951), Pike (1962), and Baird-Parker (1963).

Evans (1955) in the 7th edition of Bergey's Manual (1957), Mossel (1962), Pike (1962), Baird-Parker (1963, 1965a) and Rosypal et al. (1966) use the characters of fermentation and oxidation of glucose in a complex medium as criteria for placing strains in the genera Staphylococcus and Micrococcus. Pohja (1960), however, defines his Micrococcus strains as being both fermentative and oxidative.

The utilisation of other carbohydrates

The number of carbohydrates, which can be used in a classification scheme of micrococci and staphylococci, appears to be limited. Shaw *et al.* (1951) investigated the action of their strains on 13 "sugars" but found that apart from glucose only a few of these "sugars" were of any taxonomic use. After having tried 28 carbohydrates, Baird-Parker (1963) recommended the use of only glucose, mannitol, lactose, starch and aesculin, the last 2 being utilised by a very few strains. Apart from glucose, mannitol is the one sugar to be used by many taxonomists, and some consider fermentation of mannitol to be an important diagnostic test for pathogenic or coagulase positive staphylococci (Evans, 1947, 1948; Mossel, 1962; Baird-Parker, 1963), whereas others do not recognise any significance in its utilisation (Colwell, 1939; Shaw *et al.* 1951; Thatcher & Simon, 1957).

The oxidation of carbohydrates by washed respiring organisms

The oxidation of carbon compounds by washed, respiring cells of micrococci or staphylococci has not been studied to a large extent, but Nunheimer & Fabian (1942), using manometric techniques, found that *Micrococcus luteus*, *M. flavus*, *M. aurantiacus*, *M. cinnabareus* and *M. freudenreichii*, oxidised glucose, maltose and sucrose. Rosypal & Kocur (1963), also using manometric techniques, found that washed cells of 13

strains of *Micrococcus luteus* oxidised glucose, fructose, maltose, sucrose and galactose.

Although these two results show clearly that the *Micrococcus* species, *M. luteus*, *M. freudenreichii*, and *M. aurantiacus* do utilise glucose, Baird-Parker (1965a) placed them into his *Micrococcus* subgroup 7, which he defined as a group containing strains which either "do not release detectable acid when carbohydrates are utilised, or produce only small amounts of acid from these substrates".

Since Baird-Parker (1965a) admitted that the composition of the basal media used for detecting carbohydrate utilisation, i.e. by acid production, is important, and different media can give different results (Cowan & Steel, 1964), it may well be that acid production from "sugars" by bacteria is not the best way to detect if the organism can utilise the carbohydrate. Therefore, it is possible that too great an emphasis is placed on the character of acid production from glucose in a complex medium under anaerobic conditions, considering that this reaction is, at present, the sole criterion for separating strains of *Staphylococcus* from strains of *Micrococcus*.

Production of Acetoin

Voges & Proskauer (1898) observed that some bacterial cultures growing in a glucose medium when made alkaline with potassium hydroxide, developed a red colouration on standing. Harden (1906) found that acetoin was produced and this substance would give a red colour with alkali in the presence of peptone. The detection of acetoin was considered a useful test (the V.P. reaction) in the identification of certain groups of bacteria, and improvements in carrying out the test were made; the methods of Barrit (1936) and Batty-Smith (1941) are those used today.

Abd-el-Malek & Gibson (1948), Shaw *et al.* (1951), Pike (1962) and Baird-Parker (1963, 1965a) used acetoin production as a main criterion in their respective classification schemes of micrococci and staphylococci. Shaw *et al.* (1951) pointed out that if a very sensitive method was used, as that of Batty-Smith (1941), the reaction could lose its differential value. These authors also noted that more positive results were obtained from an unbuffered medium than from a phosphate buffered one, a point also noted by Baird-Parker (1963). He recommended that cultures should be incubated for at least 14 days before testing for the presence of acetoin. Eddy (1961), nevertheless, stated that with some bacteria the V.P. reaction may weaken with prolonged incubation and with others may even become negative. This is probably due to the partial

or total conversion of acetoin into compounds which do not give a positive V.P. reaction, i.e. 2:3 butanediol. In the case of Staphylococcus aureus, however, Watt & Workman (1951) point out that 3 times as much acetoin is produced as 2:3 butanediol, and therefore it seems unlikely that all the acetoin will be converted to other compounds, at least within the staphylococcus group.

Despite the findings that (a) different media for acetoin production and different acetoin-detecting methods may result in a greater or lesser number of positive results in one batch of strains (Shaw et al. 1951; Eddy, 1961; Baird-Parker, 1963); that (b) the possibility of "false negatives", owing to the conversion of acetoin to 2:3 butanediol, may occur (Eddy, 1961); and that (c) acetoin may be produced by two different biochemical pathways (De Ley, 1959), which would constitute "two distinct taxonomic features irrespective of the identity of the end product" (Eddy, 1961), the production of acetoin from glucose is still used as a main character for classifying micrococci and staphylococci.

Nitrate Reduction

The nitrate reduction test has been used by practically all taxonomists in the classification of micrococci and staphylococci, and it is generally agreed that most fermentative staphylococcal strains, and some oxidative micrococcal strains, reduce nitrate; only a few of the micrococci unable to utilise glucose were able to reduce nitrate (e.g. Shaw et al., 1951; Pohja, 1960; Baird-Parker, 1963). Unfortunately, nitrate reduction has proved to be of little value as a main character for classifying strains into groups or subgroups, and taxonomists tend to ignore the results when they are characterising their respective groupings.

Catalase Production

Catalase activity was recorded in bacteria in 1903 by Lowenstein and in 1923 by McLeod & Gordon. Yet it was not until 1948 that the catalase reaction of micrococci and staphylococci was found to be taxonomically significant. In this year Isaacs & Scouller reported that 146 strains of Staphylococcus, Micrococcus and Sarcina were all catalase positive and 116 strains of streptococci were all catalase negative, and although Shaw et al. (1951) did not quote these authors, the classification scheme of Shaw et al. (1951) involved only "aerobic catalase-positive Gram-positive cocci". Hucker (1954) and Evans (1955), in the 7th edition of Bergey's Manual (1957), mention that species in the genera Staphylococcus

and *Micrococcus* are catalase positive, but the catalase activity of species in the genera *Gaffkya* and *Sarcina*, also in the family *Micrococcaceae*, is not mentioned.

Kocur & Martinec (1962), Pohja (1960), Anderson (1962), Mossel (1962) and Baird-Parker (1963, 1965a) agreed that catalase activity is a fundamental criterion of strains in the micrococcus-staphylococcus group, but Hill (1959) and Pike (1962) do not mention the possession of this enzyme as a character in their respective classification schemes.

Shaw *et al.* (1951) noted a feebly catalase positive group of Gram-positive cocci, which was later characterised and given the name *Aerococcus* by Williams *et al.* (1953), but this group is considered separate from the micrococcus - staphylococcus group, although it may have some affinity towards the genus *Gaffkya* (Deibel & Niven, 1960).

Oxidase Activity

The ability of a bacterial species (*Pseudomonas pyocyanea*) to produce an oxidase enzyme was first recorded by Kovacs in 1956. Pohja (1960) found that 14% of his micrococcal and staphylococcal strains oxidised Kovacs' reagent, tetramethyl-p-phenylenediamine, and Steel (1961) observed that only one of 97 staphylococcal strains were oxidase positive. Eddy (personal communication to K.J. Steel, 1962b) reported that a negative oxidase reaction was "characteristic of *Staph*.

aureus and Staph. saprophyticus and a slow positive reaction occurred with some strains of Staph. afermentans, Staph. lactis and Staph. roseus" (Nomenclature of Shaw et al., 1951)"; Eddy also recommended the addition of 0.1% ascorbic acid to Kovacs' reagent in order to retard the autooxidation of the latter compound. Using Eddy's modification of Kovacs' (1956) method, Steel (1962b) confirmed Eddy's original findings, that Staphylococcus aureus and S. saprophyticus were oxidase negative, and that some strains of S. lactis, S. afermentans and S. roseus (nomenclature of Shaw et al., 1951) were oxidase positive.

Steel (1962b), one of the few taxonomists who examined micrococci and staphylococci for oxidase production, could not find any correlation between oxidase activity and other biochemical reactions. Although Steel (1962b) stated that he could not comment on the taxonomic significance of oxidase activity in micrococci and staphylococci, he showed that with reference to glucose metabolism "strains showing oxidase activity generally appeared to be oxidisers rather than fermentors".

Anderson (1962) used Kovacs' (1956) method for testing the oxidase reaction of 205 strains of marine micrococci, but he found that all his strains were oxidase negative - this finding could be attributed to the fact that results with the original

method are less easy to interpret than results from the modification (Steel, 1962b).

Proteolytic Action

The enzymic degradation of various types of protein has been regarded as an important character in the taxonomy of staphylococci and micrococci from some of the earliest work, (e.g. Andrewes & Gordon, 1907) to the classification schemes of today (e.g. Rosypal et al., 1966). The most widely studied substrates for proteolysis are milk or casein and gelatin.

Milk clearing and casein hydrolysis

Hastings (1904), Abd-el-Malek & Gibson (1948), Pike (1962) and Baird-Parker (1963), have regarded clearing of milk in milk agar plates as synonymous with casein hydrolysis, provided the plates are flooded with acidic mercuric chloride (Frazier, 1926) to distinguish between true proteolysis of casein and clearing of the milk, owing to the dissolving of the milk protein by alkaline end products of metabolism; this was first pointed out by Hastings (1904). Anderson (1962) did not add any acidic mercuric chloride to precipitate out the residual protein, and therefore his results cannot be compared with those of the above authors.

Abd-el-Malek & Gibson (1948) and Pike (1962) considered that most micrococcal and staphylococcal strains (including those of Staphylococcus aureus) were unable to hydrolyse casein.

Anderson (1962) found that only 36% of his strains were able to clear milk and that less than a third of these strains were able to ferment glucose. Baird-Parker (1963), however, noticed that most of his glucose fermenting strains produced clearing in milk agar. In recent years, neither clearing of milk nor casein hydrolysis has proved to be of any value in classifying micrococci and staphylococci.

Breakdown of gelatin

Visible liquefaction of gelatin in tubes has been used by many taxonomists in the classification of micrococci and staphylococci (Andrewes & Gordon, 1907; Hucker, 1924a,b; Abd-el-Malek & Gibson, 1948; Shaw *et al.*, 1951; Hill, 1959; Pohja, 1960). The papers of Abd-el-Malek & Gibson (1948) and Shaw *et al.*, (1951) point out that they had difficulty in deciding how long to incubate tubes before recording negative reactions.

Hydrolysis of gelatin can also be detected in an agar plate; when this is done the modification of Frazier (1926) (page 49) is invariably used. Many taxonomists prefer the plate method which gives a clear-cut positive or negative result (Mossel, 1962; Anderson, 1962; Kocur & Martinec, 1962; Baird-Parker, 1963).

Most coagulase-positive staphylococci break down gelatin (Cowan, 1938; Baird-Parker, 1963), but many other Gram positive, catalase positive cocci including micrococci and staphylococci

do so also (Cowan, 1938; Shaw *et al.*, 1951; Kocur & Martinec, 1962; Baird-Parker, 1963). Although it is frequently used as a character in classification schemes, gelatinase production cannot be correlated with any other character (Elek, 1959).

Hydrolysis of Urea

Shaw *et al.*, (1951) preferred the feebly buffered but sensitive urea medium of Christensen (1946) to the highly buffered medium of Stuart *et al.* (1945). Pohja (1960) and Mossel (1962) also used Christensen's (1946) medium.

Winslow *et al.* (1920), Utzino *et al.* (1938) and Hill & White (1941) have shown that the majority of *Staphylococcus aureus* strains hydrolyse urea, and more recently the percentage of positive results recorded by Shaw *et al.* in 1951 (89%), Krynski *et al.* in 1962 (86%) and Mossel in 1962 (80%) support this statement. Many coagulase negative micrococci and staphylococci also hydrolyse urea but the percentages of positive results by the following authors are not in close agreement - Shaw *et al.* 1951 (43%), Fusillo & Jaffurs, 1955 (87%), Pohja, 1960 (46%) and Mossel, 1962 (61%).

Shaw *et al.* (1951), Pohja (1960) and Mossel (1962) are the only taxonomists in recent years who have used hydrolysis of urea as a character in their respective classifications of micrococci and staphylococci, but these workers found that there was no correlation between urease production and any other

character shown by the bacteria.

Lipase Activity

Lipase activity has not been generally considered to be a very important criterion in the taxonomy of micrococci and staphylococci, and the taxonomists who have used this character in classifying this group of bacteria are listed in Table 11. Agar media containing 11 different fats were chosen by the 7 authors to demonstrate lipase activity by strains of micrococci and staphylococci (Abd-el-Malek & Gibson, 1948 did not give the constituents of their "tributyrin" medium).

It is interesting to note that Baird-Parker (1963) used an egg yolk medium for demonstrating proteolysis, but makes no mention of opaque zones, which could indicate lipase activity; nor does Pike (1962) compare the results he obtained from tributyrin agar with those from egg yolk. Yet in 1952 Gillespie & Alder thought that egg yolk opacity was caused by a lipase, and in 1959 Jessen *et al*. found a close correspondence between a positive egg yolk reaction and the ability to hydrolyse Tween 80. It seems from the results of the authors listed in Table 11 that for a wide range of staphylococcal and micrococcal strains the breakdown of tributyrin occurs more readily than the lipolysis of other fats or the opacification of egg yolk. Breakdown of fat by staphylococci and micrococci is not mentioned in the 6th or 7th editions (contributors

Table 11

Lipolysis by micrococci and staphylococci: comparison of media used

Authors	Medium	Results
Abd-el-Malek & Gibson, (1948) Classification Scheme - Table 2	1) 5% fat in nutrient agar 2) tributyrin	Strains gave the same results on the two media in their groups II and III, but more +ves were recorded on tributyrin in group I.
Hill (1959)	Tributyrin agar	Not mentioned
Pohja (1960)	11% arachis oil in meat extract agar	25% (42 out of 171) of strains lipolytic. His subgroups a, b, k, & l were non-lipolytic; the other subgroups variable.
Anderson (1962)	10% cream in a basal agar medium	50% of his strains were lipolytic.
Pike (1962) Classicication Scheme - Table 4	1) Tributyrin agar 2) Egg yolk agar.	His groups I, III, IV all broke down tributyrin. Group II failed to do so. Production of opacity in egg yolk was limited to a few staphylococci in Group I.
Baird-Parker (1963) Classification Scheme - Table 5	Lard, butter and Tweens 20, 40, 60 and 80 in agar.	All substrates were hydrolysed by most isolates which utilised glucose anaerobically (staphylococci) Tween 80 was the fat attacked least. The other organisms (micrococci) did not generally split the fats.

in Table 1) of Bergey's Manual (1948;1957) or in the papers of Shaw *et al*. (1951) and Mossel (1962).

Coagulase Production

Introduction

Although Loeb (1903) recorded that goose plasma was clotted by *Staphylococcus aureus*, Much (1908) was the first person to notice the uniqueness of coagulase. His conclusions were that only pathogenic staphylococci produce the enzyme, a fact that subsequent research workers not only confirmed but used in a test that could differentiate pathogenic Gram positive cocci, *Staphylococcus aureus*, from morphologically similar organisms (Cruickshank, 1937; Chapman *et al*.,1938; Gillespie *et al*.,1939; Christie & Keogh, 1940).

Much (1908) noted that staphylococci which clotted plasma were also clumped by it. Duthie (1954a,b) called the factor causing the clumping "bound" coagulase to differentiate it from the clotting factor which he called "free" coagulase. The production of "free" coagulase can be determined by the tube test (Fisk, 1940). The clumping property of "bound" coagulase has been used by Cadness-Graves *et al*. (1943) in their slide test, which can be used for the rapid identification of "free" coagulase positive staphylococci. Despite the fact that Cadness-Graves *et al*. (1943) found a close correlation between the results of the tube test and the slide

test, Smith (1962), examining strains from dogs, found that slide tests gave fewer positives than did tube tests; both Elek (1959) and Morrison (1962) considered that 10% of weakly coagulase positive (tube test) strains are not detectable by the slide test.

Taxonomists classifying micrococci and staphylococci in recent years appear to have used mainly the tube method of Fisk (1940)(Shaw et al.,1951; Pohja 1960; Baird-Parker 1963). The slide test is a very useful tool for a rapid routine diagnosis of coagulase positive Staphylococcus aureus, but in critical taxonomic studies the tube test is a more accurate method of detecting coagulase producing organisms.

The association of coagulase with pathogenicity

Although Much (1908), Daranyi (1927), Chapman et al. (1934), Cruickshank (1937) and Fairbrother (1940) all claimed that coagulase production is associated with pathogenicity, Elek (1959) considered that this statement is a generalisation and "clearly a positive finding only reveals a potentiality for pathogenicity". Foster (1962) stated that although coagulase is associated with strains from lesions, the enzyme does not play any part in staphylococcal infections.

In addition, a very few coagulase negative staphylococci and micrococci have been reported as pathogenic. Cunliffe et al. (1943) discovered a coagulase negative staphylococcus

that caused bacterial endocarditis and recently 2 new species of pathogenic micrococci, which do not produce coagulase, have been discovered, Micrococcus hyicus, causative organisms of contagious impetigo in pigs (Sompalinsky, 1953), and Micrococcus violagabriellae, an organism responsible for a human chronic skin infection (Castellani, 1955).

Therefore, the claim made by early medical bacteriologists that all pathogenic strains of the Micrococcaceae must be coagulase positive is no longer true.

Phosphatase Production

Barber & Kuper (1951) found that a certain degree of correlation existed between phosphatase and coagulase production of staphylococci, and thus they considered that phosphatase production by a Gram positive coccus was indicative of the organism being a coagulase producing Staphylococcus aureus. These authors grew their organisms on nutrient agar plates containing phenolphthalein diphosphate at 37°C for 18 hours, and then exposed the plates to ammonia vapours; phosphatase producing colonies turned deep pink on exposure to the ammonia.

Hill (1959), Pike (1962) and Kocur & Martinec (1962) used this phosphatase test in their classifications of micrococci and staphylococci, but Kocur & Martinec (1962) prolonged the time of incubation to 48 hours before exposing plates to the ammonia. When Jacobs et al. (1964), however, examined 333 strains of staphylococci for phosphatase production after

incubation of the cultures at 37°C for 18 hours, they found that phosphatase activity was of no value in discriminating between coagulase positive staphylococci and coagulase negative staphylococci since many of the latter also produced phosphatase.

Baird-Parker (1963) used the phosphatase reaction as one of the criteria for his group division, but he lengthened the incubation time to 3-5 days and maintained the temperature at 30°C. Baird-Parker (1963) found that all coagulase positive isolates were strongly phosphatase positive, and 402 of 570 coagulase negative staphylococci were also phosphatase positive; a very few strains of micrococci showed phosphatase activity, but the reaction was often weak. Classifications previous to 1951, of course, make no mention of this test, but some later authors involved in the taxonomy of staphylococci and micrococci also do not refer to it (Pohja, 1960; Anderson 1962; Mossel 1962; Jones _et al._,1963). There is no doubt that the length of incubation time influences the number of positive reactions, and this time factor must be taken into consideration when phosphatase production is used as a criterion in any taxonomic scheme.

Haemolysin Production

Practically all coagulase positive staphylococci are known to produce haemolysins but there is little agreement among taxonomists concerning haemolysin production by coagulase negative staphylococci or micrococci.

Coagulase positive strains

Three serologically distinct haemolysins are produced by strains of coagulase positive staphylococci; the α-haemolysin, acting on rabbit and, to a lesser extent, on sheep, human and horse red cells, (Glenny & Stevens, 1935); the hot-cold β-haemolysin, acting on sheep and human, but not rabbit red cells (Glenny & Stevens, 1935), and the δ-haemolysin acting on sheep, rabbit, human and horse red cells (Williams & Harper, 1947). Elek & Levy (1950b) considered that the γ-haemolysin of Smith & Price (1938b) was identical to the δ-haemolysin.

Elek & Levy (1950b), using a plate method with sheep and rabbit red cells, found that one of the 3 haemolysins, α-, β- or δ-, was produced by all coagulase positive strains and very often all 3 were produced. On the other hand, Thatcher & Simon (1957) could not find any complete correlation between coagulase production and α- or β-haemolysin production or phage susceptibility. Abd-el Malek & Gibson (1948) mentioned that 321 out of 328 coagulase positive staphylococci were "haemolytic" and apparently, although this is not clearly stated, produced "darkened zones" on sheep and ox blood agar after 48 hours' incubation at the strains' optimum temperature and then left overnight in a refrigerator. From this information it is possible that these 321 strains were β-haemolytic. Pike (1962) examined 326 strains of micrococci and

staphylococci on 10% human blood agar for haemolysis after incubation for 18 hours at the strains' optimum temperature and found that all his 40 coagulase positive strains were, in fact, "haemolytic", but he gives no more details about the type of haemolysin produced.

Coagulase negative strains

Coagulase negative staphylococci and micrococci do not produce α-, β- or δ-haemolysins, and when investigated for haemolysin production have been found to be negative (Chapman et al., 1934; Cowan, 1938; Williams & Harper, 1947). Abd-el-Malek & Gibson in 1948 however showed that 12 out of 113 coagulase negative staphylococci were "haemolytic", but none of these strains produced the presence of "darkened zones" on sheep and ox blood agar, unlike the coagulase positive strains (page 57). It is probable that these 12 strains were not β-haemolytic.

Elek & Levy (1950b) stated that 95% of all the 77 coagulase negative micrococcal and staphylococcal strains they investigated produced a haemolysin unneutralised by α-, β- or δ-anti-toxins and haemolytic for all species of red blood cells. They designated this haemolysin ϵ. Hill (1959) included the production of α-, β-, δ- and ϵ-haemolysins as criteria for his Adansonian classification of staphylococci, but he did not discuss the results. His only comment is that

Staphylococcus aureus usually produces α-lysin, whereas *Staphylococcus saprophyticus* and *Micrococcus roseus* do not. Pike (1962) found that 102 out of 296 coagulase negative strains were "haemolytic", but does not describe the haemolysis produced. Therefore, there is still relatively little known about the nature or taxonomic significance of ϵ-haemolysin.

Many taxonomists do not use the haemolysis of blood as a character in the classification of staphylococci and micrococci (Shaw *et al.*, 1951; Mossel, 1962; Kocur & Martinec, 1962; Anderson, 1962; Baird-Parker, 1963; Rosypal *et al.*, 1966). Some of those that do, forget to mention the type of blood in their experiments (Van Eseltine, 1955; Pohja, 1960; Kocur & Martinec, 1963b), an omission critisised strongly by Elek & Levy (1950b) and Cowan (1962).

COMPARISON OF THE MAIN MORPHOLOGICAL AND PHYSIOLOGICAL CLASSIFICATION SCHEMES OF MICROCOCCI AND STAPHYLOCOCCI FROM 1948 TO 1965

This comparison is restricted to non-Adansonian classification schemes, in which certain selected characters are used as criteria for placing strains of micrococci and staphylococci into species, groups or subgroups - the Adansonian classification schemes are reviewed on page 91.

Before 1955, acid production from glucose by Gram positive, catalase positive cocci had been used as a character for classifying these bacteria, but no mention had been made as to whether the glucose had been oxidised or fermented. After this date, glucose fermentation was the standard criterion in morphological and physiological classification schemes for dividing the genus *Micrococcus* from the genus *Staphylococcus* (page 14). Consequently, it is difficult to compare the results of taxonomists before and after this date; the comparison is made even more difficult by the fact that the only characters shared in all the classification schemes before and after 1955 are acid and acetoin production from glucose, and nitrate reduction. I will compare, therefore, the 3 classification schemes previous to 1955 separately from those published after this date.

Pre-1955 classification schemes (Tables 1, 2, 3 and 12)

The classification schemes of Hucker (1943) and Hall (1944) in the 6th edition of Bergey's Manual (1948), Abd-el-Malek & Gibson

Table 12

Comparison of morphological and physiological classification schemes of micrococci and staphylococci between 1948 and 1951

Hucker (1943) and Hall (1944) in 6th edition of Bergey's Manual (1948)	Abd-el-Malek & Gibson (1948)	Shaw et al. (1951)
Micrococcus pyogenes	*Staphylococcus* Group IA	Subgroup 1 (*Staphylococcus aureus*)
Micrococcus epidermidis	*Staphylococcus* Groups IB, IC and ID	Subgroup 2 (*S.saprophyticus*)
?	Dairy *Micrococcus* Groups IIIA and IIIB	Subgroup 3 (*S.lactis*)
?	Intermediate Group: Group II	Subgroup 5 (*S.afermentans*)
Micrococcus Roseus	-	Subgroup 4 (*S.roseus*)

(1948), and Shaw *et al.* (1951) share the following characters as criteria for grouping - pigment production, acid and acetoin production from glucose, and coagulase production. The species, groups and subgroups are compared in Table 12.

Post-1955 classification schemes (Tables 1, 4, 5 and 13)

Anderson (1962) did not try to classify his strains of marine micrococci, although he arranged them in 16 undefined groups; Mossel (1962) was mainly interested in distinguishing between *Staphylococcus aureus* and *Staphylococcus saprophyticus*, and he found that coagulase production was the only character of use in separating the 2 species. I, therefore, do not include the classification schemes of Anderson (1962) and Mossel (1962) in this comparison (Table 13).

The classification schemes of contributors (Table 1) to 7th edition of Bergey (1957), Pike (1962) and Baird-Parker (1965a) used the same following characters as criteria for grouping - pigment production, glucose and mannitol fermentation, oxidation of glucose and coagulase production. Kocur & Martinec (1962) who classified only *Micrococcus* species, naturally did not examine strains for the characters of carbohydrate fermentation or coagulase production, but their scheme used pigment production and oxidation of glucose as main characters for grouping.

It is worth noting here that Baird-Parker (1965a) examined some of Abd-el-Malek & Gibson's (1948) group III strains, and

Table 13

Comparison of morphological and physiological classification schemes of micrococci and staphylococci between 1957 and 1965

Contributors (Table 1) in 7th edition of Bergey's Manual (1957)	Kocur & Martinec (1962)	Pike (1962)	Baird-Parker (1963, 1965a)
Staphylococcus aureus	-	Staphylococcus Group IA	Staphylococcus Subgroup I
Staphylococcus epidermidis	-	Staphylococcus Group Ib	Staphylococcus Subgroup II
Staphylococcus epidermidis	-	Staphylococcus Group IC	Staphylococcus Subgroup IV or V
?	-	?	Staphylococcus Subgroup III and VI
Micrococcus caseolyticus ?	Micrococcus varians ?	Staphylococcus Group Id and Micrococcus Group IIIb	Micrococcus Subgroup 5
?	?	?	Micrococcus Subgroup 6
?	?	Micrococcus Group IIIa	Micrococcus Subgroups 1, 2, 3 or 4
Sarcina lutea	Micrococcus luteus	Sarcina Group IV	Micrococcus Subgroup 7
Micrococcus roseus	Micrococcus roseus	-	Micrococcus Subgroup 8

Baird-Parker (1965a) considered that group IIIA was the same as Baird-Parker's (1963, 1965a) *Micrococcus* subgroup 5, but that strains of Abd-el-Malek & Gibson's (1948) group IIIB, because of their unusual morphological and physiological characters, were not included in the family *Micrococcaceae*.

From the information presented in Tables 12 and 13, the only groupings that are similar in all these classification schemes are (1) the coagulase positive, glucose fermenting staphylococci (*Staphylococcus aureus*), (2) the yellow pigmented micrococci unable to oxidise glucose (*Micrococcus luteus*), and (3) the red pigmented micrococci unable to oxidise glucose (*Micrococcus roseus*).

There is, however, no agreement concerning the taxonomic position of the other groupings. I consider that this lack of agreement is due to the selection by these taxonomists of main characters for classifying these strains into groups, before the taxonomists found if these characters were typical of the groups they had just created. As these main characters were selected by the arbitrary choice of the taxonomists, it is not surprising that there is little agreement between the groupings in the classification schemes.

In my opinion, it is essential that an Adansonian approach to classification should be adopted, andthen see if any

SEROLOGICAL CHARACTERS

Serological typing of strains within the family Micrococcaceae is limited to coagulase positive organisms and even here "the antigenic properties of Staphylococcus aureus have been considered difficult to explore. Methods which were used with success on other bacteria failed with staphylococci. There has been a general feeling that the antigenic structure of this organism is so complex that a systemic classification can hardly be achieved." (Ooding, 1960).

The different methods of serological typing and the results obtained are listed in Table 14. Early work, based on agglutinations and agglutinin absorption, could do little more than differentiate between the pathogenic and the saprophytic strains with any consistency (Kolle & Otto, 1902; Hine, 1922). The precipitation tests of Julianelle & Wieghard (1935), Thompson & Khorazo (1937) and Cowan (1938) showed that there were 2 main groups among the coagulase positive strains and 2 main groups also among the saprophytic strains. However, up till 1939 many coagulase positive strains could not be sero-typed. The work of Cowan (1939), Christie & Keogh (1940) and Hobbs (1948) produced 3 main and 10 subtypes and allowed serological typing of Staphylococcus aureus to be performed on a world-wide scale but, although Brodie (1957) referred to these 13 subtypes as "international types", Cowan (1962) stated that "no international body is authorised

Table 14

Different techniques of serological typing and the results obtained by various authors

Authors	Methods of serological typing	Results
Kolle & Otto (1902)	Agglutination	Pathogenic staphylococci different from saprophytic ones
Julianelle (1922)	Agglutinin absorption	3 serological groups among 25 strains of *S. aureus*
Hine (1922)	Agglutinin absorption	2 groups: 1) *S.pyogenes* with 3 types 2) *S.epidermidis albus* with 2 types
Seedorf (1924)	Complement fixation	13 types of *S.aureus*
Julianelle & Wieghard (1935)	Precipitation of carbohydrate antigens	2 groups: 1) A "apparently virulent strains" - *S.aureus* 2) B "Avirulent" strains - *S.albus*
Thompson & Khorazo (1937)	Precipitation of carbohydrate antigens	additional group to those of Julianelle & Wieghard (1935) among the saprophytic strains
Cowan (1938)	Precipitation of carbohydrates antigens	additional group to those of Julianelle & Wieghard (1935) among the coagulase +ve strains
Cowan (1939)	Slide agglutination of boiled organisms	3 types and an "atypical" group among *S.aureus* strains
Christie & Keogh (1940)	Slide agglutination of boiled organisms	9 types among *S.aureus* strains
Hobbs (1948)	Slide agglutination of boiled organisms	13 types among *S.aureus* strains
Mercier *et al.* (1950)	Slide agglutination of boiled strains	3 types among *S.aureus* strains (Cowan types)

Table 14 (continued)

Authors	Methods of serological typing	Results
Andersen (1943)	Agglutinin absorption of heat treated and trypsin digested organisms	antigens detected a) heat stable b) trypsin stable & heat labile c) trypsin labile & heat labile
Oeding (1952b)	Serum prepared from formalin-killed strains; slide agglutination with live strains.	9 types among 540 strains of *S. aureus*
Oeding (1953c)	Agglutinin absorption of live and heat killed strains.	10 antigens demonstrated (a-k excluding j) and used for a typing scheme of *S. aureus*
Oeding & Williams (1958)	Comparison of serological types based on antigens a-k and phage type	Broad correlation between serological typing and phage typing
Cowan (1938)	Animal strains-precipitation of carbohydrate antigens	Coagulase +ve strains belong mainly to Group A of Julianelle & Wieghard (1935)
White et al. (1962)	Animal strains - Agglutinin absorption	3 groups A B C out of 502 strains (coagulase +ve) distinct from the 13 serotypes of Cowan (1939), Christie & Keogh (1940), and Hobbs (1948)

to grant recognition to staphylococcus serotypes". Oeding's work (1952b, 1953c) identified 10 antigens which formed the basis for his typing scheme and Oeding & Vogelsang (1954), were thus able to type 94.6% of hospital coagulase positive strains.

Although Cowan (1938) could place coagulase positive strains from animals into Julianelle & Wieghard's (1935) Group A, White *et al.* (1962) found that coagulase positive animal strains were grouped separately (Table 14) from the 13 serotypes of Cowan (1938), Christie & Keogh (1940) and Hobbs (1948). Oeding (1960) considered that the antigenic differences observed in animal and human strains may be related to host species. It may well be that serological characters can change with environment.

The serology of coagulase negative staphylococci and micrococci has not yet been fully investigated.

PHAGE TYPING CHARACTERS

Development of *Staphylococcus* phage typing

An important development occurred in the taxonomy of *Staphylococcus aureus* when Fisk (1942b) found that most strains of this organism were lysogenic. From this observation, he began a crude phage typing system by cross culturing many strains and looking for lysis. Wilson & Atkinson (1945) isolated a series of phages for phage typing; *Staphylococcus aureus* strains were typed by patterns of lysis occurring when 18 phages were placed on a lawn of growth of each strain.

Williams & Ripon (1952) extended the range of phages used in typing and also noted that weak reactions of phage on cultures would have to be taken into account.

Coagulase positive strains

Over 90% of *Staphylococcus aureus* strains are phage typable and they can be placed into one of the four phage groups. Other strains of *Staphylococcus aureus* that are lysed by phages belonging to 2 or more groups cannot be placed into any of the phage groups (Parker, 1962).

Phage groups I, II and III correspond approximately to Cowan's serological groups I, II and III (Hobbs, 1948; Parker, 1962). Oeding & Williams (1958) also found a close correlation between phage types and Oeding's (1952b, 1953c) serological types.

Within the phage groups, lysis by certain phages is associated with the ability of the strains to give rise to particular clinical conditions, e.g. the 52, 52A, 80, 81 complex of phage type within Group I causes severe sepsis in hospital patients (Parker, 1962).

Staphylococcus aureus strains isolated from cows or milk are very often lysed by only one phage (42D), which would place the strains in the miscellaneous group, otherwise known as Group IV, (MacDonald, 1946; Smith, 1948a). Human strains rarely belong to Group IV (Parker 1962). This may be a useful character in differentiating between bovine and human types.

Staphylococcus aureus strains from sheep appear to be mostly untypable with human phages but may be subdivided with phages isolated from ovine staphylococci (Smith, 1948b). Thus, there can be relations between the phage pattern of a strain and its environment.

Coagulase negative strains

Although there are phages which act on coagulase negative strains of micrococci and staphylococci, these phages seem, so far, to be of little use in distinguishing species within this group (Cowan, 1962). As yet, however, phage typing of coagulase negative micrococci and staphylococci has not been fully examined.

ECOLOGICAL CHARACTERS

The science of ecology can be defined as the study of organisms in relation to their surroundings. Strains of the micrococcus-staphylococcus group of organisms have been studied from a variety of habitats and many of the recent classification schemes of this group were in fact evolved as the result of limited knowledge of micrococci and staphylococci from a particular habitat. For instance, before they presented their classification scheme, Abd-el-Malek & Gibson (1948) found that "difficulties were immediately encountered when attempts were made to identify staphylococci and micrococci isolated from milk", and on discussing strains of Gram positive, catalase positive cocci present in the human mouth, Pike (1962) found that "no recent taxonomic study of the oral Micrococcaceae appears to have been made and this may be the cause of inaccurate nomenclature, common in dental literature, which impedes comparison of different authors' findings". Pike (1962) then went ahead and presented his own classification scheme for oral staphylococci and micrococci.

Although Shaw *et al.* (1951) did not think that the source of a culture should have any place in a bacterial classification scheme, they and all the taxonomists listed in Table 15 mentioned at least some details of the sources of their strains. Ecological information of strains within the family Micrococcaceae is shown in Table 15. I have summarised this

information and this, with some additional material, is given below:-

(1) Coagulase positive staphylococci:-

Coagulase positive staphylococci are found in the human mouth, human and animal skin, and are often associated with infections in man and animals. Coagulase positive staphylococci from human sources can usually be distinguished from those on animal sources by the haemolysins produced and phage typing.

(2) Coagulase negative staphylococci:-

Coagulase negative staphylococci are frequently found in the human mouth and human and animal skin and may occasionally occur in dairy products. There is little evidence for associating coagulase negative staphylococci with disease, but recently two different infections were found to be caused by two coagulase negative glucose fermentors. One strain, *Micrococcus hyicus* was responsible for an outbreak of contagious impetigo of swine (Sompalinsky, 1953) and the other, *Micrococcus violagabriellae*, for a chronic human skin infection (Castellani, 1955).

(3) Micrococci:-

Micrococci are the predominant members of the *Micrococcaceae* in such habitats as air, soil, water, cured and fermented meat products, and milk and dairy produce. *Micrococcus* species, as defined by Evans, 1955, in 7th edition Bergey's Manual 1957, are not known to cause disease.

Table 15

Ecological findings of authors since 1948 who have studied the taxonomy of micrococci and staphylococci.

Authors	Main sources investigated	Ecological results
Abd-el-Malek & Gibson (1948) Classification Scheme - Table 2	cows' udders, raw milk, pasteurised milk, sheep skin, human skin and infections	Strains from human and animal bodies belong to their Staphylococcus group I. Strains from milk and dairy equipment belong to their heat resistant dairy micrococci group III.
Elek & Levy (1950b)	Human and animal skin and infections.	Coagulase + ve strains: α- and δ- haemolysins occurred very frequently in human strains, β-haemolysins characteristic of animal but not human strains. Coagulase - ve strains: human and animal skin strains either produce haemolysin or are non-haemolytic.
Shaw et al. (1951) Classification Scheme - Table 3	Human, dogs, sheep, cows, milk, ice-cream, dairy utensils, food, air, water, soil and sewage.	"Source of a culture should not have a place in any bacterial classification scheme". Most strains fell into Shaw et al. (1951) subgroupings 1, 2 and 3. Sources of all subgroup 4 strains not known and sources of only a few of subgroup 5 strains known.
Thatcher & Simon. (1956)	Clinical sources and cheese and butter	Coagulase + ve strains; strains from clinical sources - phage group III common, 42D rare. Strains from dairy products - mostly 42D group IV of bovine origin. Coagulase - ve strains: more micrococci

Table 15 (continued)

Authors	Main sources investigated.	Ecological results
Thatcher & Simon (1956)	Clinical sources and cheese and butter	isolated from dairy sources than from clinical ones.
7th edition of Bergey's Manual (1957); for contributors see Table 1.	1) skin, milk, dairy utensils, cheese, frozen meat, dust, water and salt lakes. 2) nasal and skin sources 3) human respiratory tract and diseased lobsters 4) air, soil, dust, mud, sewage, sea water and wheat bran.	16 species of Micrococcus came from sources in 1). 2 species of Staphylococcus came from sources in 2) 2 species of Gaffkya came from sources in 3). 10 species of Sarcina came from sources in 4.
Pohja (1960)	Fermented meat products and curing brine.	Most strains belong to S. lactis subgroup 3 of Shaw et al. (1951) and are in 1 of 3 further subdivisions; halophilic fermentative micrococci, halotolerant fermentative micrococci and non-halotolerant fermentative micrococci.
Kitchell (1962)	Raw, cured and fermented meat products, (coagulase +ve strains excluded from study)	Pork and bacon microfloras related. Majority of isolates belong to Shaw et al. (1951) subgroup 3 (S. lactis)
Sharpe et al. (1962)	Cows' udders, milk and cheese.	Coagulase + ve strains: cause mastitis in cows. Coagulase - ve strains on udders, in cheese and milk.

Table 15 (continued)

Authors	Main sources investigated.	Ecological results
Pike (1962)	Human and animal mouths, saliva and carious teeth.	Staphylococci dominant members of *Micrococcaceae* in human mouth. *S. lactis* found occasionally in carious teeth but not saliva. *Sarcina lutea* occasionally in saliva but not in carious teeth. *Micrococcus* spp. not found in human mouth. *Gaffkya tetragena* found only in human saliva and carious teeth.
Anderson (1962)	North Sea south-east of Aberdeen at 10 m.	Most strains "fermentative" and many proteolytic. No conclusions drawn betweem enviroment and Anderson's subgroupings.
Baird-Parker (1965a)	Human nose and skin, fermented meats, bacon, brine, pork, air, soil and sea.	Coagulase - ve strains only: Staphylococci predominant in human nose and skin, especially Baird-Parker (1963,1965a) subgroups II and VI. Micrococci predominant in the air (especially *Micrococcus* subgroup 3), soil, meat and dairy produce.

(4) Gaffkya species:-

Gaffkya tetragena is invariably found only in the human mouth and respiratory tract. Gaffkya homari occurs in diseased lobsters, but was thought by Coster & White (1964) to be an Aerococcus species, and therefore outwith the family Micrococcaceae.

(5) Sarcina species:-

Sarcinas are known to occur in habitats similar to those in which micrococci live.

Although some information is known about the ecology of Gram positive, catalase positive cocci, evidence has not been put forward by any taxonomist that, for instance, micrococci found in milk and milk products may be more likely to utilise lactose than micrococci found in soil or water. In other words, no research work has been done to see if any physiological character can be associated with the occurrence of an organism in a particular habitat.

THE USE OF EXTRACTABLE CELL COMPONENTS AS CHARACTERS FOR CLASSIFICATION

A relatively new approach to try to solve the problem of classifying strains of micrococci and staphylococci is that of the study of the chemical nature of cell components - acid hydrolysis products of cell walls; sugars, amino sugars, and amino acids (Cummins & Harris, 1956a,b; Pike, 1962; Baird-Parker, 1965a): lysozyme hydrolysis products of cell walls; amino acids (Salton & Pavlik, 1960): acid extractable teichoic acids from cell walls and whole cells (Davison & Baddiley, 1963; Losnegard & Oeding, 1964a,b; Baird-Parker, 1965a): acid extractable amino acids from whole cells (Gregory & Mabbit, 1957; Baird-Parker, 1965a).

Cell Wall Sugars, Amino Sugars and Amino Acids

Cummins & Harris (1956b) examined acid hydrolysates of cell wall preparations of 60 strains of Gram positive bacteria, including 16 strains of catalase positive cocci, by the technique of paper chromatography. They analysed these hydrolysates for the presence of sugars, amino sugars, and amino acids, and divided the 16 strains of Gram positive, catalase positive cocci into 3 groups on their cell wall composition.

Into group 1 they placed 9 strains of the organisms *Staphylococcus aureus*, *S. albus*, *S. citreus*, *Micrococcus luteus* and *Sarcina lutea*; cell walls of these strains contained the

same amino acids, amino sugars and sugars with the exception of 2 constituents, glucose and serine (Table 16), but Cummins & Harris (1956a) considered that further division within this group was not easy to justify on the basis of the results they obtained.

Into group 2 they placed 1 strain of Micrococcus conglomeratus and 4 strains of Aerococcus viridans. Cummins & Harris (1956a) formed this group as distinct from group 1, since the strains in group 2 contained galactosamine but not glycine in their cell walls (Table 16). I find this grouping difficult to agree with since, first, when I looked at their results (Cummins & Harris, 1956a) I found that only 2 of these strains (2 Aerococcus sp.) had identical cell wall composition and, secondly, they do not distinguish between the catalase negative or weak Aerococcus sp. and the strongly catalase positive Micrococcus sp.

Since the third group consisted of 2 named species of Micrococcus, which were in fact diphtheroids, as they already knew (Shaw et al., 1951) and admitted (Cummins & Harris, 1956a), Baird-Parker (1965a), quite correctly, rejected it from any taxonomic study involving Gram positive, catalase positive cocci. Pike (1962) and Baird-Parker (1965a) also examined some of their strains of micrococci and staphylococci by the cell wall analysis technique of Cummins & Harris (1956a). The results of the 3 papers are summarised in Table 16. On the whole,

The results of cell wall analysis of strains of the family Micrococcaceae

Authors	No of strains examined	Groupings	Principal components used for classification			
			Glucose	Serine	Glycine	Galactosamine
Cummings & Harris (1856a)	9	Group 1 Staphylococcus aureus (3) *	—	Tr	+	—
		S.albus (3)	—	+	+	—
		S.citreus (1)	+	—	+	—
		Micrococcus luteus (1)	+	—	+	—
		Sarcina lutea (1)	+	—	+	—
	5	Group 2 Micrococcus conglomeratus (1)	+	—	—	Tr
		Aerococcus viridans (4)	+	—	—	+
Pike (1962) (for physiological classification see Table 4)	19	Group Ia (3)	N.L.	Tr	+	N.L.
		Groups Ib, c, d (3)	N.L.	+	+	N.L.
		Groups II (5) and IIIb (2)	N.L.	+	+	N.L.
		Group IV (6)	N.L.	—	+	N.L.
Baird-Parker (1965a) (for physiological classification see Table 5)	46	Subgroup I (3)	—	+	+	—
		Subgroup II (6)	+	+	+	—
		Subgroups III (3) IV (3), V (2) and VI (6)	±	+	+	±
		Subgroups 1 (3), 2 (3), 3 (3) 4 (1), 5 (3) and 6 (2)	+	+	+	+

* Numbers in brackets = strains

Table 16 (continued)

Authors	No. of strains examined	Groupings	Principal components used for classification			
			Glucose	Serine	Glycine	Galactosamine
Baird-Parker (1965a)	46	Subgroup 7 (3)*	+	±	+	+
		Subgroup 8 (3)	+	-	-	+
		Subgroup Sarcina (2)	+	-	+	+

Numbers in brackets = strains Tr = trace N.L. = not looked for

\+ = present ± = present in small amounts - = absent

Pike (1962) and Baird-Parker (1965a) confirm the work of Cummins & Harris (1956a), but Baird-Parker (1965a) found that galactosamine was present in the cell walls of all micrococci, a result that Cummins & Harris (1956a) did not show.

It seems that cell wall analysis is of its greatest taxonomic value, within the Gram positive, catalase positive cocci, in the separation of members of the Micrococcaceae from other coccus-forming groups of bacteria. Within the micrococcus-staphylococcus group, however, classification by this technique is limited by the homogeneity of the composition of the cell walls (Pike, 1962; Baird-Parker, 1965a), although it is possible to distinguish between 2 groups of organisms within the Micrococcaceae on the basis of the presence or absence of serine in the cell walls; the red and yellow pigmented micrococci unable to utilise glucose, do not have serine, whereas other micrococci and staphylococci contain this amino acid in their cell walls. Similarly, strains of *Micrococcus roseus* do not have cell wall glycine, whereas other Gram positive, catalase positive, cocci possess this amino acid in their cell walls.

Relative Molecular Proportions of Cell Wall Amino Acids

Salton & Pavlik (1960) treated cell wall preparations of strains of Gram positive bacteria with egg white lysozyme, and they examined the resultant cell wall hydrolysis products. Table 17 shows the results that they obtained for 12 strains of Gram

Table 17

Relative molecular proportions of the principal amino acids in cell walls of strains within the aerobic *Micrococcaceae*

Organism *	Baird-Parker (1965a) subgroup	Lysine	Glutamic acid	Glycine	Serine	Alanine
Staphylococcus albus N.C.T.C. 7942	unknown	1	1.1	4.8	0.8	2.9
S. aureus	unknown	1	1.1	4.8	0.9	3.0
S. citreus	unknown	1	1	4	1	3.1
S. saprophyticus	unknown	1	1	4.6	1.2	3.3
Micrococcus citreus N.C.T.C. 7731	unknown	1	3	0.8	0	2.1
M. lysodeikticus N.C.T.C. 2665	*Micrococcus* subgroup 7	1	1	1	0	2.6
M. tetragenus	*Micrococcus* subgroup 7	1	1.2	1.2	0	2.3
M. urea	unknown	1	1.3	1	0	2.3
Sarcina flava	*Micrococcus* subgroup 7	1	1.4	1	0	2.2
Sarcina lutea	*Micrococcus* subgroup 7	1	1.6	1	0	2.0
M. roseus	*Micrococcus* subgroup 8	1	1.1	0	0	5.1
Sporosarcina ureae	unknown	1	1.7	1	0	2.0

* = Nomenclature of Salton & Pavlik (1960)

positive, catalase positive cocci. Salton & Pavlik (1960) did not comment on the taxonomic significance of their results. In addition, the sources of only 3 of the strains were given by Salton & Pavlik (1960), and since they did not try to classify their strains by physiological tests, their results cannot be strictly compared with the results of other taxonomists. Certainly Baird-Parker (1965a) was not justified in assigning 5 of the 12 species of Salton & Pavlik (1960) into Baird-Parker (1965a) groupings, since he merely deduced from the generic and specific names the appropriate groupings (Table 17). Nevertheless, it is possible to say, from the results of Salton & Pavlik (1960), that first, the cell walls of their _Staphylococcus_ species contain about 4 times as much glycine as the cell walls of their _Micrococcus_, _Sarcina_ and _Sporosarcina_ species; secondly, only the _Staphylococcus_ species contain serine in the cell walls (pages 72 & 73); and thirdly, the cell walls of their _Micrococcus roseus_ species differ from those of other _Micrococcus_ species in that they contain no glycine (page 73) and twice as much alanine.

From the results presented in Table 17, I find a close correlation between the relative molecular proportions of the five amino acids of _Sporosarcina ureae_, and those of all the other _Micrococcus_ species, with the exception of _Micrococcus roseus_. Yet other information makes it more probable that the genus _Sporosarcina_ is more closely related to the family

Bacillaceae than to the family Micrococcaceae (MacDonald, 1962; Kocur & Martinec, 1963b, page 18). Thus, in my opinion, more work will have to be done before any definite conclusion can be drawn about the worth of relative molecular proportions of cell wall amino acids in taxonomic studies of the family Micrococcaceae.

Teichoic Acids

Teichoic acids are found intracellularly in all micrococci and staphylococci, but are only present in the cell walls of some of these organisms (Davison & Baddiley, 1963). These authors considered that a classification of staphylococci could be evolved on the basis of teichoic acid composition, and proposed a division of strains into 3 groups, one group containing strains with ribitol teichoic acid in their cell walls, another group containing strains with glycerol teichoic acid in their cell walls, and a third group containing strains with no teichoic acid in their cell walls. Further work by Losnegard & Oeding (1963a,b), Davison *et al.*,(1964) and Davison & Baddiley (1964) on the teichoic acid composition of staphylococcal cell walls by chemical, enzymic, and serological means has extended the number of recognisable groups from 3 to 6, the characters of which are summarised in Table 18. Baird-Parker (1965a) reported that variable amounts of teichoic acids were present in the cell walls of most strains, but were not detected in walls of strains within the biochemically

Table 18

Composition and characters of the teichoic acids obtained from some staphylococci according to Davison & Baddiley (1964), Davidson et al. (1964) and Losnegard & Oeding (1963a, b).

Strains	Teichoic acid		Composition of wall teichoic acid		Serological carbohydrate antigens
	Intra-cellular	Wall	Polyol	Sugar	
Staphylococcus aureus (most strains)	G	R	Ribitol	mixture of both α- & β- N-acetyl glucosamine	linkage corr-esponds to poly-saccharide A of Wieghard & Julianelle. (1935)
Staphylococcus aureus (few strains)	G	R	Ribitol	only one type of linkage of N-acetylglu-cosamine either α or β	
intermediate strains between S. aureus and S.epidermidis (coagulase-ve)	G	R	Ribitol	mainly β- (some α-) N-acetyl-glucosamine	polysaccharide AC
S. epidermidis-saprophyticus group 1)	G	G	Glycerol	α-glucosyl residue	probably poly-saccharide B
2)	G	G	Glycerol	β-glucosyl residue	unknown
3)	G	-	-	-	-

G= glycerol teichoic acid R = ribitol teichoic acid

inactive *Micrococcus* subgroups 7 and 8 (Table 5). It seems to me that in cell wall hydrolysates the absence of teichoic acids correlates well with the absence of serine (page 73).

Acetic Acid - Extractable Amino Acids and Peptides from Whole Cells

Mattick *et al.* (1956) were the first taxonomists to show that chromatographic analysis of acetic acid extracts of whole cells were of use in classifying bacteria. Using the same technique, Gregory & Mabbit (1957) examined representative species of cocci, classified according to the scheme of Shaw *et al.*, 1951 (Table 3). Although Gregory & Mabbit (1957) found that *Staphylococcus aureus* was distinguished from *S. saprophyticus* by patterns of ninhydrin-staining amino acids and peptides, they were unable to differentiate between *S. lactis*, *S. roseus* and *S. afermentans*. Baird-Parker (1965a), using the same technique, examined 46 of his micrococcal and staphylococcal strains; he found 2 main patterns. One pattern was typical of "most staphylococci", members of *Micrococcus* subgroups 1-3 and some members of *Micrococcus* subgroups 5 and 6 (Table 5). The other pattern was shown by the majority of strains of *Micrococcus* subgroup 5 and all the strains belonging to *Micrococcus* subgroups 7 and 8. Although the use of this technique, like that of cell wall analysis (page 71) seems to be a limited one for the classification of strains within the *Micrococcaceae*, it may be useful for rejecting organisms

from the family, as when Gregory & Mabbit (1957) found that the difference between the pattern of amino acids and peptides of strains of Abd-el-Malek & Gibson (1948) groups IIIA and IIIB (Table 2) was greater than between the type strains of Staphylococcus aureus and S. saprophyticus. Taking into account these results and his own findings, Baird-Parker (1965a) considered that group IIIB organisms of Abd-el-Malek & Gibson (1948) are not members of the Micrococcaceae.

THE MOLECULAR BIOLOGICAL APPROACH TO CLASSIFICATION

One of the most important and recent developments in the taxonomic field is the use of the structure of the bacterial DNA molecule as a criterion for classification - the use of the structure of bacterial DNA for taxonomic purposes is called the molecular biological approach to classification. At present the taxonomic interest lies with (a) the ratio of guanine to cytosine, 2 bases on the DNA molecule, for different bacteria, (b) the base sequence of different bacterial and viral DNA molecules, and (c) the degree of homology between DNA molecules of different strains.

DNA Base Ratios

The taxonomic significance of DNA base ratios in the family Micrococcaceae

Lee et al. (1956) were the first people to study DNA base ratios of organisms as a basis for classifying them. They calculated the ratio $\frac{\text{adenine + thymine}}{\text{guanine + cytosine}}$ for DNAs of 60 strains of bacteria, including 8 members of the family Micrococcaceae, and although Lee et al. (1956) did not work out the moles % guanine + cytosine in total DNA bases (% GC ratios), which subsequent taxonomists studying DNA base ratios of micrococci and staphylococci have done, I could easily calculate % GC ratios from their tabulated results.

Bohacek et al. (1965) and Auletta & Kennedy (1966) have also examined DNA base ratios of Gram positive cocci and compared

their % GC results with physiological classificationsof the same strains.

The results of all these authors are summarised in Table 19. Bohacek *et al.* (1965) and Auletta & Kennedy (1966) have confirmed the earlier work of Lee *et. al.* (1956) on the % GC ratios of strains of the genera *Staphylococcus*, *Micrococcus* and *Sarcina*, and have also shown that most strains of Gram positive cocci fit into 2 large but distinct groups of % GC ratios:- one group contains strains which have a low % GC ratio (30-39) and this corresponds to the genus *Staphylococcus* as re-defined by Evans (1955); the other group contains strains which have a high % GC ratio (59-75.5) and this corresponds to the genus *Micrococcus* (Hucker, 1954, Breed, 1954) and *Sarcina* (Smit, 1949).

The small anomalous groups, shown in Table 19, of Bohacek *et al.* (1965) and Auletta & Kennedy (1966), contain strains, which are no longer considered to be members of the *Micrococcaceae*: *Pediococcus* (Breed, 1955), *Sporosarcina ureae* (MacDonald, 1962, Mazanec *et al.* 1965).

Silvestri & Hill (1965a) were the first taxonomists to compare a classification of Gram positive catalase positive cocci by numerical taxonomic (or in Silvestri & Hill's, 1965a, terminology - "taxometric") methods with a classification of

Table 19

The relationship between DNA base ratio classifications and morphological and physiological classification schemes as found by various authors.

Authors	Strains examined	Classification schemes compared	Results
Lee et al. (1956)	8 strains of Staphylococcus, Micrococcus and Sarcina	None compared	5 Staphylococcus aureus strains with %GC ratios 30.7 - 32. 1 Micrococcus lysodeikticus strain with a %GC ratio 71.9 1 Sarcina flava strain with a %GC ratio of 68.6, and 1 Sarcina Lutea with a %GC ratio of 63.9
Bohacek, et al. (1965)	31 strains of Staphylococcus, Micrococcus and Pediococcus	The morphological and physiological classification of Kocur & Martinec (1962) and DNA base ratios	2 main groups:- 1) 6 strains with GC values 30.2 - 35.6 % fermented glucose belong to the genus Staphylococcus 2) 23 strains with GC values 65.5 - 75.5% did not ferment glucose and belong to the genus Micrococcus 1 small anomalous group:- contained 1 strain M.cryophilus and 1 strain of Pediococcus with GC values of 41.3 - 44.4 %
Auletta & Kennedy (1966)	37 strains of Staphylococcus, Micrococcus, Gaffkya and Sarcina	A morphogical & physiological classification and DNA base ratios	2 main groups:- I) 17 strains with GC values 30 - 39% fermented dextrose - Staphylococcus aureus - epidermidis-violagabriellae group II) 19 strains with GC values 59 - 72% did not ferment dextrose - Micrococcus & Sarcina group 1 small anomalous group:- 1 strain Sporosarcina ureae with GC values 43% - excluded from Micrococcaceae

the same strains on the basis of DNA base ratios. Silvestri & Hill (1965a) examined 13 strains of Staphylococcus (nomenclature of Shaw et al. 1951), which had been previously classified by Hill (1959) and Hill et al. (1965) by taxometric methods (page 91), and came to the conclusion that the division of Gram positive, catalase positive cocci into 2 groups by % GC ratios could be correlated with the division of the same strains into 2 main groups by taxometric methods (Table 20).

Rosypal et al. (1966) also compared a classification scheme of strains of micrococci and staphylococci based on % GC ratios with a numerical taxonomic method of classifying the same strains. They divided 37 strains into 7 DNA base ratio groupings, which Rosypal et al. (1966) then further divided into 10 subgroups by similarity values calculated from the results of the numerical taxonomic method of classification (Table 20). One other subgroup (3c) contained 1 strain of the species Micrococcus denitrificans, and on the evidence of Baird-Parker (1965a), who considered that the species was more closely related to "Gram negative genera" than to the Micrococcaceae, Rosypal et al. (1966) rejected it from their classification scheme of micrococci and staphylococci.

Whereas the results of Silvestri & Hill (1965a) are in agreement with those of Bohacek et al. (1965) and Auletta & Kennedy (1966) in that micrococci and staphylococci can be

Table 20

The relationship between DNA base ratio classifications and Adansonian classifications

Authors	Strains examined	Classification schemes compared	Results
Silvestri & Hill (1965a)	13 strains of *Staphylococcus* (nomenclature of Shaw *et al* 1951)	Taxometric methods of analysis of morphological and physiological characters and DNA base ratios	2 main groups: 1) 8 strains with low % GC ratio (30.8 - 36.5) belong to the *Staphylococcus aureus-saprophyticus-lactis* taxometric group which corresponds to the genus *Staphylococcus*. 2) 5 strains with high % GC ratio (69- 75) belong to the *Staphylococcus roseus-afermentans* taxometric group which corresponds to the genus *Micrococcus*,
Rosypal *et al*. (1966)	37 strains of *Staphylococcus*, *Micrococcus*, and *Sarcina*	Taxometric method of analysis of morphological and physiological characters and DNA base ratios	7 groups divided up on % GC ratios; 10 subgroups divided up on similarity values calculated from the Adansonian analysis.
			group 1 (70.8 - 73.3 % GC) containing 2 subgroups - 1a (16 strains) and 1b (4 strains).
			Group 2 (67.5 - 69.5 % GC) containing 3 subgroups - 2a (5 strains), 2b and 2c each with 1 strain.
			group 3 (66.3 - 67 % GC) containing 3 subgroups - 3a, 3b, and 3c each with 1 strain.

Table 20 continued.

The relationship between DNA base ratio classifications and Adansonian classifications

Authors	Strains examined	Classification schemes compared	Results
Rosypal et al. (1966)	37 strains of *Staphylococcus*, *Micrococcus*, and *Sarcina*	Taxometric method of analysis of morphological and physiological characters and DNA base ratios.	group 4 (54.2 % GC) containing 1 strain.
			group 5 (36.4 % GC) containing 1 strain.
			group 6 (33.3-34.2%GC) containing 3 strains.
			group 7 (30.7-34.2%GC) containing 2 strains.
			groups 1, 2, and 3 belong to the genus *Micrococcus*
			groups 5, 6 and 7 belong to the genus *Staphylococcus*
			group 4 contains only 1 species, *Sarcina* [illegible] - its taxonomic position in the family *Micrococcaceae* is not yet known.

divided into 2 main groups, the classification scheme of Rosypal *et al.* (1966) is substantially different in that it contains 7 main groups.

Although I consider Rosypal *et al*'s. (1966) division into groups and subgroups too fine from the results that they obtained (see criticism of results below) they claim that their scheme resembles that of Baird-Parker (1965a) - Table 5 - at least in the *Micrococcus* groupings.

Very recently Hill (1966) produced an index of % GC ratios of many bacterial species from culture collections. He has tabulated the % GC ratio results of various authors of 69 named species of 3 genera in the *Micrococcaceae* and these results are expressed graphically in Table 21. Hill (1966) did not discuss or comment on these results. The names of the species listed under *Micrococcus* are as follows:-

M. afermentans (5)*, *M. aquivivus* (1), *M. asaccharolyticus* (1), *M. conglomeratus* (3), *M. cryophilus* (1), *M. denitrificans* (2), *M. eucinetus* (2), *M. halodenitrificans* (2), *M. litoralis* (1), *M. luteus* (8), *M. lysodeikticus* (3), *M. radiodurans* (3), *M. roseus* (7), and 2 unknown *Micrococcus* species.

Even if the species, which are no longer considered to be members of the genus *Micrococcus*, *M. cryophilus* (Bohacek *et al.*, 1965; (page 34), *M. denitrificans* (Verhoeven, 1957; Baird-Parker, 1965a; (page 32), *M. halodenitrificans* (Robinson & Gibbons, 1952; (page 32), *M. radiodurans* (Work, 1964; (page 32) are removed from the above list, the corresponding % GC ratios

* Numbers in brackets = strains

Table 21

Hill's (1966) DNA base ratio results for the 3 genera in the family *Micrococcaceae* expressed graphically

Moles % guanine + cytosine (%GC) in total DNA bases

25 30 35 40 45 50 55 60 65 70 75

41 *Micrococcus* spp.

10 *Sarcina* spp.

24 *Staphylococcus* spp.

expressed in Table 21 are little altered, and strains of Micrococcus can be found with % GC ratios of 34, 45-51, 53.5-58.5 and 63.5-75.5. Thus, Hill (1966) has shown that the genus Micrococcus contains strains with a much wider range of % GC ratios than had previously been thought (Tables 19, 20 & 21).

The names of the listed Sarcina species (Hill, 1966) are as follows:- S. aurantiaca (1)*, S. flava (2), S. lutea (4), S. ureae (3).

If Sarcina (Sporosarcina) ureae is removed from the genus Sarcina on the evidence of MacDonald (1962), Kocur & Martinec (1963b) and Mazanec et al. (1965) (pages 18&75), the other listed Sarcina species contain a relatively narrow range of % GC ratios of 64-74.

The results of % GC ratios of Sarcina species shown by Hill (1966), therefore, agree with those of the taxonomists listed in Table 19, in that the range of Sarcina % GC ratios falls within the range of Micrococcus % GC ratios and that strains within the genus Sarcina cannot be distinguished by DNA base ratios from strains within the genus Micrococcus (see also page 79).

Although most taxonomists, e.g. Shaw et al. (1951), Hill (1959), Pike (1962) and Hill et al. (1965), recognise the species listed by Hill (1966) under Staphylococcus - S. aureus (17)*, S. lactis (3) and S. saprophyticus (4) - as belonging to that genus, Pohja (1960) and Baird-Parker (1965a) considered

* Numbers in brackets = strains.

that strains of S. lactis should be placed in the genus Micrococcus. Whichever taxonomic viewpoint is taken over the generic position of S. lactis, all but one of the strains (S. lactis N.C.T.C. 7564) listed by Hill (1966) fall into a relatively narrow % GC ratio (30-40).

It does seem, however, from the results presented by Hill (1966) since the 3 strains listed have 32, 36.5 and 69 % GC ratios respectively, that S. lactis could be a heterogeneous group, a view held by Gregory & Mabbit (1957), Hill (1959), Pohja (1960) and Baird-Parker (1963), and, in addition, Hill et al. (1965) showed that this anomalous strain of S. lactis is more related to the Micrococcus roseus - afermentans group of organisms than to the Staphylococcus aureus - saprophyticus - lactis group by numerical taxonomic methods (Table 22). Therefore if the DNA base ratio results of this one strain (N.C.T.C. 7564) are excluded from the % GC ratio results of the Staphylococcus sp. presented by Hill (1966), the results are in agreement with those of the taxonomists listed in Tables 19 and 20, in that strains of Staphylococcus sp. have a low percentage of guanine and cytosine in their DNA.

Thus in reviewing all the results of DNA base ratio studies of micrococci and staphylococci, I find that there is evidence at present to suggest that strains can belong to one of two groups depending on their % GC base ratios, but the amount of work does not justify a finer division, and certainly the % GC

ratios of strains of Micrococcus species must be examined further before any true classification scheme can be devised for the Micrococcaceae on the basis of DNA base ratios. It is possible that as more strains of Gram positive, catalase positive cocci are examined, some strains will be found to have DNA base ratios intermediate between those of staphylococci (30-40 % GC) and micrococci (65-73 % GC).

Criticism of results

There are two points of criticism I would level against the five classification schemes listed in Tables 19,20, & 21.

(1) In all these schemes relatively few strains were examined. If, say, 200 strains were to be examined by the grouping and subgrouping arrangement of Rosypal et al. (1966), it is highly likely that many more groups and subgroups would have to be created to classify all the strains which would possess a wide range of DNA base ratios and many differing physiological characters. Rosypal et al. (1966) found that only one of their subgroups contained more than 5 strains, and in fact 7 out of their 12 groupings and subgroupings contained only one representative. Many more strains will have to be investigated before it is possible to say that a DNA base ratio classification is a considerable advance in the field of micrococcal and staphylococcal taxonomy.

(2) The source of strains for all these taxonomic studies is, in practically every case, culture collections. Strains from

culture collections are not always typical of the species they represent, and also strains cultured for a long time may lose certain characters and this means that % GC ratios must alter; in fact, it has been shown by Gause _et al._ (1964) that a strain of a staphylococcus, with a DNA base composition of 32.4 % GC, on ultra violet irradiation produced several mutant strains each with a DNA base composition of 69.2-71.0 % GC. Therefore, because recently isolated strains may have sufficiently different base ratios not to fit into any of the groupings listed in Tables 19, 20 and 21, in the future at least some of recent isolates should be examined in any taxonomic study by DNA base ratios.

I am also critical of the undue emphasis that has been placed on DNA base ratios in recent years for the classification of bacteria. The composition of DNA of strains from the bacterial families so far examined may vary from about 25% guanine + cytosine total bases to about 75% GC (Silvestri & Hill, 1965b). Almost the complete range of these GC% values can be found in the aerobic members of the _Micrococcaceae_ alone. It would be reasonable to assume that a classification scheme, based on DNA base ratios alone, of all bacterial species, would differ fundamentally from all recognised classification schemes. For instance, _Streptomyces_, _Myxococcus_, and many strains of _Micrococcus_ have a narrow GC % base ratio range of 65.5 to 73.5 (Hill, 1966). Does this mean that strains of

these 3 genera should all be placed in one group or family? Very few taxonomists would agree with this arrangement.

DNA base ratios, therefore, can give only a limited amount of information since they are not related directly to the functions of organisms.

When more information about the DNA of micrococci and staphylococci becomes readily available, the molecular-biological approach to classification may become important in their taxonomy.

Nearest Neighbour Base Sequence Analysis of DNA

Studies of nearest neighbour base sequences in DNA by Josse *et al*. (1961) and Swartz *et al*. (1962) showed that 16 times as much information could be obtained about the base sequences of DNA of cells by this technique as by DNA base ratio analysis. This information is likely to be used in future classifications, and therefore nearest neighbour base sequence analysis may open the way for many new taxonomic applications. Certainly Morrison *et al*. (1967) have used this analysis to determine the extent of resemblance of DNA of 3 mammalian viruses to the DNA of their host cells, and these authors at the moment are examining the relatedness of strains of bacteria by the same technique.

DNA Homology

In 1963, McCarthy & Bolton determined the degree of DNA homology of an *E. coli* strain with strains of other Gram

negative bacteria by a DNA hybridization method. They took a known weight of sheared and denatured E. coli DNA and trapped it in an agar gel. The DNA was unable to renature but was still available for complementary interaction with free nucleic acid molecules. Radioactively labelled DNA from other strains were also sheared so that the DNA molecules were able to penetrate the agar gel and react with the trapped DNA. Excess DNA was washed off and then McCarthy & Bolton (1963) calculated the percentage of labelled DNA bound to the E. coli DNA and therefore determined the degree of DNA homology. They found, for instance, that Salmonella typhimurium (1)* and Shigella dysenterieae (1) are more related to E. coli than Aerobacter aerogenes (2) or Klebsiella pneumoniae (1) by this DNA hybridization method of classification.

De Ley (1965) examined strains of Pseudomonas and Xanthomonas by the same method.

Although De Ley (1965) does not give much information on his Pseudomonas DNA hybridization results, he does mention that "DNA homology corroborates species-differentiation in this genus". He also considered that Xanthomonas should contain only one species and that since strains of the 2 genera Pseudomonas and Xanthomonas "share 45-80% of their DNA" there should be only one genus, Pseudomonas, which would contain

* Numbers in brackets = strains.

strains for both groups of organisms.

In addition, he examined DNA base composition of the strains and found that, since % GC ratios of strains of *Pseudomonas* overlapped considerably the % GC ratios of *Xanthomonas* strains, the base ratio results supported the above conclusion, based on DNA homology, that strains at present in the genera *Pseudomonas* and *Xanthomonas* should not be separate genera but be contained in one group under the generic name *Pseudomonas*.

It seems to me that DNA hybridization experiments would be well worth carrying out in the *Micrococcus-Staphylococcus* group of organisms. It is interesting to note that De Ley (1965) compared similarity values of strains analysed by a numerical taxonomic method with DNA homology results of the same strains, and he deduced from this comparison that all DNA is used, at least in the *Pseudomonas-Xanthomonas* group, for phenotypic expression. I would expect, therefore, a classification of strains of the family *Micrococcaceae*, based on DNA hybridization experiments, to be similar to classification schemes based on Adansonian analysis of morphological and physiological characters (Table 22).

THE ADANSONIAN APPROACH TO CLASSIFICATION

Introduction

Very little is known, at present, about the biochemistry of the metabolic pathways used by bacteria to give positive reactions in physiological tests, andit is not possible, for instance, to calculatetho number of enzymes that are involved in the demonstration of one character. Therefore, it is reasonable to assume that, until more knowledge is available, every character should be given equal importance for classification purposes. This concept should apply not only to physiological characters but to all other characters, e.g. morphological, DNA base composition serological and electrophoretic characters. The first taxonomist who considered using every character impartially and with equal weight was Michel Adanson (1763); classification schemes based on such a concept are referred to as Adansonian (Sneath, 1957a). Sneath (1957a,b, 1962) advocated the Adansonian approach to bacterial classification, in which each of the equally weighted characters would be handled mathematically to calculate affinities between strains. This method of analysing results is now called numerical taxonomy (Sneath & Sokal, 1962), and the analysis is most rapidly and efficiently carried out by computer, as first proposed by Sneath (1957b). The procedure for using numerical taxonomy and its applications are discussed by Sokal & Sneath (1963), but a brief outline of

the method is presented here.

First of all, a large number of characters must be chosen, preferably over 60, which are representative of as many aspects as possible of the strains to be classified. Secondly, the measure of affinity between 2 strains is determined by comparing characters shared and not shared; various coefficients of similarity can be used to measure this affinity. The range of similarity coefficients extends from unity for complete agreement to zero for no agreement whatsoever. The actual calculations of the coefficients of similarity are tedious and time-consuming if carried out by hand, and therefore a computer should be used if it is available. Thirdly, the coefficients of similarity are tabulated in a matrix form for every pair of strains, so that natural groupings or clusters can easily be observed, and from this matrix diagram a taxonomic hierachy of the strains can be designed, usually in the form of a dendrogram. The clustering from the matrix diagram can be carried out manually, or by an automatic method with the use of a computer.

If an identification scheme is desired, this can be constructed from the Adansonian classification scheme by the selection of certain characters that are typical of all the strains classified, and also those that are typical of the naturally occuring clusters. These characters are used to form a simple dichotomous key.

Table 22

Adansonian classification schemes of micrococci and staphylococci

Authors	Strains	Characters compared	Results
Hill (1959)	49	80	Staphylococcus aureus, (20), S.saprophyticus (8), & S. roseus (7) formed natural taxonomic groups. S. lactis (19) and S.afermentans (5) did not.
Pohja (1960)	191	62	S. lactis (164) could be divided into 10 Adansonian groups, which were subdivided by tolerance to 0 & 15 % NaCl. S. aureus (3), S.saprophyticus (12), & Micrococcus roseus (4) formed natural taxonomic groups.
Pohja & Gyllenberg (1962)	2 test groups (I & II) each of 62	62	Halophiles (3) formed a distinct group but S.saprophyticus (5) not a distinct group by itself. Non-halotolerant strains (13) in a heterogeneous group. Non-fermentative strains (2) also heterogeneous. 6 related subgroups within halotolerant S. lactis. group (62).
Rosypal et. al. (1966)	37	67	7 micrococcus and 3 staphylococcus subgroups. Each subgroup comprised the strains with approximately the same DNA base composition and a high % similarity of physiological and biochemical characters.

Nos. in brackets = strains

<u>The Adansonian approach to the taxonomy of micrococci and staphylococci</u>

The results of 4 papers relating to Adansonian classification schemes of micrococci and staphylococci are listed in Table 22. The results of Hill (1959) and Pohja (1960), both using the same coefficient of similarity, show agreement in that strains of <u>Staphylococcus aureus</u>, <u>S. saprophyticus</u> and <u>S. roseus</u> (nomenclature of Shaw <u>et al</u>., 1951; (page 23) fall into 3 distinct groupings corresponding to the 3 species. Hill (1959), however, found that strains of <u>S. lactis</u> and <u>S. afermentans</u> (nomenclature of Shaw <u>et al</u>., 1951; (page 23) did not fall into natural taxonomic groups, and he considered that these 2 species were artificially created, since Shaw <u>et al</u>. (1951) had defined them largely on negative criteria. Gregory & Mabbit (1957) and Baird-Parker (1965a) also considered that <u>S. lactis</u> is a heterogeneous group of strains, and Pohja (1960) divided strains of this species into 10 groupings by numerical taxonomic means. Pohja & Gyllenberg (1962) used 3 different coefficients of similarity to examine some of Pohja's (1960) original strains; the results were the same for the 3 coefficients - that strains of <u>S. lactis</u> can be placed into 1 of 5 subgroups or into a miscellaneous group of heterogeneous strains. Numerical taxonomy, therefore, has shown that <u>S. lactis</u> and <u>S. afermentans</u> are, in fact, heterogeneous groups of strains.

Hill *et al.* (1965) were more interested in comparing different techniques for analysing data by numerical taxonomic means than in producing an actual classification scheme; these authors examined the strains of Hill (1959) by 2 coefficients of similarity, and also compared manual and automatic methods of clustering. The results obtained with the 2 coefficients of similarity were essentially the same, and the automatic method of clustering was found to be quicker, more objective and also allowed an easier recognition of taxonomically important characters than the manual method.

Rosypal *et al.* (1966) went one stage further and compared a numerical taxonomic method of classification with a DNA base composition classification, and they found that the 2 classification schemes were complementary to each other (for my criticism of the publication of Rosypal *et al.*, 1966 see page 84).

Attempts have been made by Hill (1959) and Pohja & Gyllenberg (1962) to devise identification schemes based on their respective Adansonian classifications, but as far as I am aware, these identification schemes have been little used by bacteriologists, who wish to identify a micrococcus or a staphylococcus. I think the main reason for the neglect of these identification schemes is that many of the groups created by these authors using numerical taxonomic means are

not generally recognised by bacterial taxonomists today.

The Adansonian approach to the taxonomy of micrococci and staphylococci gives similar results to the traditional approach to classification (page 3), and numerical taxonomy has been proved useful, for instance, in showing that *Staphylococcus lactis* is a heterogeneous group of strains. Nevertheless, so far only morphological and physiological characters have been used to classify micrococcal and staphylococcal strains by numerical taxonomic means, and in addition the strains classified have been relatively few in number. I think that there is an urgent need for a taxonomic study of several hundred Gram positive, catalase positive cocci, obtained from a wide spectrum of sources, and I consider that these strains should be examined for not only morphological and physiological characters, but also for other characters, e.g. DNA base composition, cell wall composition, and electrophoretic characters - from this broad selection of characters it should be possible to construct a first class Adansonian classification scheme acceptable to all taxonomists.

SECTION A

EXAMINATION OF THE MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERS OF 406 STRAINS OF MICROCOCCI AND STAPHYLOCOCCI

MATERIALS AND METHODS

MATERIALS AND METHODS

INTRODUCTION TO EXPERIMENTAL WORK

In view of the confusion of the taxonomy at present within the family *Micrococcaceae*, I will define the following family, generic, and specific names I intend to use in the experimental part of this thesis. The following definitions are based on the generally agreed opinions of contemporary taxonomists, which have been presented in the preceding review of the literature.

I consider that the family *Micrococcaceae* should consist of Gram positive, catalase positive cocci, and this family can be divided into 2 major groups based on the oxygen requirements of these cocci:-

(1) there are the strictly anaerobic strains - the anaerobic members of the *Micrococcaceae*, and

(2) there are the aerobic and facultatively anaerobic strains - the aerobic members of the *Micrococcaceae*. Only the second group is studied in this thesis.

The aerobic members of the family *Micrococcaceae* shall be divided into 2 genera, *Micrococcus* and *Staphylococcus*, which are defined as follows: The genus *Micrococcus* consists of strains unable to grow or produce acid from glucose under anaerobic conditions in a complex medium - non-fermentative strains.

I recognise only two species of the genus *Micrococcus* which shall be defined as follows:

The specific name *Micrococcus luteus* refers to packet forming and non-packet forming, yellow pigmented micrococci unable to utilise glucose.

The specific name *Micrococcus roseus* refers to packet forming and non-packet forming, red pigmented micrococci unable to utilise glucose.

I do not recognise any other *Micrococcus* species.

The genus *Staphylococcus* consists of strains able to produce acid from glucose under anaerobic conditions in a complex medium - fermentative strains.

I recognise only one species of the genus *Staphylococcus*, namely the type species *Staphylococcus aureus*, which is defined as being coagulase positive. Strains other than this species shall be referred to as coagulase negative staphylococci, and are not given specific names. Occasionally, however, I use the species name *S. epidermidis*, when I am comparing my results with those of other taxonomists who have described bacteria with this specific name. I define *S. epidermidis* as being coagulase negative, usually acetoin and phosphatase producing, but not able to utilise mannitol under aerobic conditions.

I do not recognise the genera *Gaffkya*, *Sarcina* nor *Sporosarcina*.

It should be noted that these definitions only apply to the

experimental sections of this thesis. In the classification section (page 305), I have revised the taxonomy of the aerobic members of the family *Micrococcaceae*, and I do not accept the generic and specific names of the family as defined on the foregoing page.

STRAINS OF MICROCOCCI AND STAPHYLOCOCCI

Origin of Strains

Over a period of 3 years, 297 strains of Gram positive cocci were obtained from a variety of sources and, in addition, 109 named micrococcal and staphylococcal strains were supplied from culture collections. The total number of strains, therefore, studied in this thesis is 406. Table 23 shows the origin of the cultures, and also the isolation media used.

Isolation of strains

The 297 strains were isolated as follows:-

181 strains were isolated by direct plating of the source material on selective media, or by streaking a damp swab, which had been thoroughly rubbed over, or soaked in, the source material, over the surface of selective media. One of the problems involved in a study of Gram positive cocci is the isolation of these organisms from a source, which may contain a mixed population of different types of bacteria and fungi; I found that Mannitol Salt Agar (Oxoid), containing 7.5% sodium chloride and 1% mannitol in a beef extract - peptone agar, was a convenient medium for the isolation of

Gram positive, catalase positive cocci, since the salt concentration is not high enough to prevent the growth of most of these organisms, but is sufficient to inhibit other bacteria from the same environment. I also used Blood Agar Base No.2 (Oxoid), containing 20% sodium chloride, to isolate a few strains of Gram positive, catalase positive cocci, which were growing in environment of high salt concentration.

91 strains were isolated by direct plating on to non-selective media of samples of clinical material from human infections (supplied to the bacteriological laboratory of the Western Infirmary, Glasgow). Since there were relatively few groups of bacteria present in the samples, I was able to pick up single colonies of the organisms I desired, after plating the specimens on the standard non-selective media used in the laboratory - Blood Agar Base No.2 (Oxoid) without the blood, and also Blood Agar Base No.2 containing 10% horse blood.

25 strains were obtained as pure cultures on nutrient agar slopes from laboratories connected with various hospitals.

The 109 named cultures were supplied from 5 culture collections as follows:-

5 strains were obtained as pure cultures on agar slopes from the Microbiology Department, University of Glasgow.

104 strains were received freeze-dried in ampoules from various culture collections (Table 23).

All the 406 strains were checked for purity by plating on

Table 23

The sources of the strains

No. of strains isolated	Isolation medium	Source		Geographical location
84	10% horse blood agar	Human	infections of human bodies	Western Infirmary, Glasgow
*8	unknown		skin lesions of humans	Dermatology Dept., University of Glasgow
*6	unknown		urinary infections of humans	Hospitals, Bristol
*2	unknown		infections of human bodies	Vale of Leven Hospital, Dumbartonshire.
*1	unknown		wound sepsis in baby	Hospital, Birmingham
66	Mannitol Salt Agar		healthy human skin	8 persons in one family, Glasgow
*8	unknown		healthy human skin	Dermatology Dept., University of Glasgow
3	Mannitol Salt Agar		healthy human skin	2 animal handlers, Microbiology Dept., University of Glasgow.
1	Mannitol Salt Agar		healthy human skin	Scottish tourist in Sitges, Spain
1	Mannitol Salt Agar	Mammals	nose of diseased rabbit	Animal House, Microbiology Dept., University of Glasgow
6	Mannitol Salt Agar		5 healthy rabbit skins	Animal House, Microbiology Dept., University of Glasgow
6	Mannitol Salt Agar		5 healthy guinea pig skins	Animal House, Microbiology Dept., University of Glasgow
7	Mannitol Salt Agar		5 healthy mouse skins	Animal House, Microbiology Dept., University of Glasgow
19	Mannitol Salt Agar		11 healthy cow skins	Slaughter House, Glasgow
3	Mannitol Salt Agar		2 healthy cat skins	Bearsden, Dumbartonshire

Table 23 (continued)

No. of strains isolated	Isolation medium	Source		Geographical location
2	Mannitol Salt Agar.		2 healthy sheep skins	Isle of Oronsay, Colonsay, Argyll
1	Mannitol Salt Agar		skin of a healthy wild hedgehog	Bearsden, Dumbartonshire.
2	Mannitol Salt Agar	Birds	2 healthy pet budgerigar skins	Bearsden, Dumbartonshire
2	Mannitol Salt Agar		2 healthy grouse crops	Glenshee, Perthshire
1	Mannitol Salt Agar		skin of a healthy sparrow	Bearsden, Dumbartonshire
2	3% NaCl Agar	Fish	decomposing fish skin	Fishmongers, Glasgow.
2	Mannitol Salt Agar		guts of a freshly caught healthy lythe (Gadus pollachius)	Isle of Oronsay, Colonsay, Argyll
2	Mannitol Salt Agar		guts of 2 freshly caught healthy saithe (Gadus virens)	Isle of Oronsay, Colonsay, Argyll
2	Mannitol Salt Agar	Crustaceans	mouth and anus of 2 freshly caught healthy live lobsters	Isle of Oronsay, Colonsay, Argyll
1	Mannitol Salt Agar	Insects	faeces of healthy caterpillars (Pieris brassicae)	Microbiology Dept., University of Glasgow.

Table 23 (continued)

No. of strains isolated	Isolation medium		Source	Geographical location
1	Mannitol Salt Agar	Food sources	raw milk	Isle of Oronsay, Colonsay, Argyll
1	20% NaCl Agar		blue cheese	Denmark, but bought in Glasgow
1	Mannitol Salt Agar		Vlaadam cheese	Belgium
41	Mannitol Salt Agar		24 assorted cheeses	France
1	Mannitol Salt Agar		bread	Sitges, Spain
1	Mannitol Salt Agar		sponge cake	Sitges, Spain
1	Mannitol Salt Agar		cream cake	Sitges, Spain
2	Mannitol Salt Agar		2 blocks of ice cream	Bought in Glasgow
1	20% NaCl Agar		vacuum packed bacon	Denmark, but bought in Glasgow
1	Mannitol Salt Agar	Other sources	sea water, surface of sea, ½ mile from shore	Isle of Oronsay, Colonsay, Argyll
1	Mannitol Salt Agar		river mud	River Kelvin, Dumbartonshire
2	10% horse blood agar		2 10/- notes	Microbiology Dept., University of Glasgow
5	Blood agar base No.2		5 aerial contaminants	Western Infirmary and Microbiology Dept., University of Glasgow

Table 23 (continued)

No. of strains isolated	Isolation medium	Source		Geographical location
*1	unknown	Culture collections	*Staphylococcus saprophyticus* 7292	N.C.T.C.
*1	unknown		*Staphylococcus citreus* 7415	N.C.T.C.
*1	unknown		*Staphylococcus afermentans* 2665	N.C.T.C.
*1	unknown		*Staphylococcus flavocyaneus* 7011	N.C.T.C.
*1	unknown		*Staphylococcus luteus* 8512	N.C.T.C.
*1	unknown		*Staphylococcus roseus* 7523	N.C.T.C.
*3	unknown		*Staphylococcus lactis* 189, 7564 and 7617	N.C.T.C.
*1	unknown		*Micrococcus violagabriellae* 9865	N.C.T.C.
*1	unknown		*Sarcina ureae* 4819	N.C.T.C.
*1	unknown		*Staphylococcus epidermidis* 8558	N.C.I.B.
*1	unknown		*Micrococcus candidus* 8610	N.C.I.B.

Table 23 (continued)

No. of strains isolated	Isolation medium	Source		Geographical location
*1	unknown	Culture Collections	*Micrococcus sodonensus* 8854	N.C.I.B.
*1	unknown		*Micrococcus radiodurans* 9279	N.C.I.B.
*2	unknown		*Micrococcus conglomeratus* 740, 836	C.C.M.
*5	unknown		*Micrococcus roseus* 146, 239, 560, 758 and 837	C.C.M.
*13	unknown		*Micrococcus luteus* 248, 291, 310, 352, 354, 365, 370, 410, 443, 531, 658, 810 and 855	C.C.M.
*5	unknown		*Staphylococcus epidermidis* 1400, 1404, 1407, 1412 and 1413	C.C.M.
*5	unknown		*Staphylococcus lactis* 02, 36, 40, 417 and 1577	C.C.M.
*1	unknown		*Micrococcus roseus*	Microbiology Dept., University of Glasgow.
*1	unknown		*Micrococcus aurantiacus*	Microbiology Dept., University of Glasgow
*1	unknown		*Micrococcus lysodeikticus*	Microbiology Dept., University of Glasgow

Table 23 (continued)

No. of strains isolated	Isolation medium	Source		Geographical location
*1	unknown	Culture collections	*Micrococcus cinnabareus*	Microbiology Dept., University of Glasgow
*1	unknown		*Sarcina lutea*	Microbiology Dept., University of Glasgow
*1	"blood" agar and tryptone yeast glucose salt agar	Baird-Parker (1963) subgroup strains	subgroup I - human skin	Dr. A. C. Baird-Parker, Bedfordshire
*11			subgroup II - human skin & mouth, pigskin and dust	
*3			subgroup III - pigskin	
*4			subgroup IV - human skin	
*3			subgroup V - human skin	
*6			subgroup VI - human and pig skin, dust and food	
*1			subgroup 1 - dust	
*4			subgroup 2 - bacon	
*9			subgroup 3 - dust, bacon	
*1			subgroup 4? - bacon	
*4			subgroup 5 - bacon and dust	

Table 23 (continued)

No. of strains isolated	Isolation medium	Source		Geographical location
*1	"blood" agar and tryptone yeast glucose salt agar	Baird-Parker (1963) subgroup strains	subgroup 5 or 6 - dust	Dr. A. C. Baird-Parker Bedfordshire
*3			subgroup 6 - pigskin and human mouth	
*4			subgroup 7 - pigskin	
*1			subgroup *Sarcina* - bacon	
*3			unclassifiable - dust	
406 Total				

N.C.T.C. = National Collection of Type Cultures, London.

N.C.I.B. = National Collection of Industrial Bacteria, Aberdeen.

C.C.M. = Czechoslovak Collection of Microorganisms, Brno.

* = obtained as a pure culture.

Blood Agar Base No.2, without any added blood, since I found that this non-selective medium was excellent for the growth of Gram positive cocci. Single colony isolates were made on to slopes of the same medium. As the strains were isolated, and before any experiments designed to find out their optimum temperature could be carried out, I chose an arbitrary temperature of incubation for the growth of the strains. I decided to incubate the strains isolated from hospital sources at 35°C, since this is a temperature at which staphylococci (the most likely group of the Gram positive, catalase positive cocci to be present in human infections) grow well, and all other strains at 30°C, since this is a temperature which taxonomists, e.g. Kocur & Martinec (1962), Baird-Parker (1963) have found to be the most suitable for the growth of both micrococci and staphylococci.

Maintenance of cultures

From the pure cultures of these 406 strains, subcultures were made on to slopes of Blood Agar Base No.2, and these were incubated at 30°C for 3 days. Initially the inoculated agar slopes were plugged with cotton wool, and to prevent dessication, liquid paraffin was poured over the surface of the slopes; when at a later stage metal caps replaced cotton wool plugs, the agar slopes did not dry out so quickly, and since subculturing from slopes covered with liquid paraffin is inconvenient, time-consuming and liable to contamination, the

use of liquid paraffin was discontinued. Instead Nutrient Broth (Oxoid) was pipetted aseptically on to the slopes until all the bacterial growth and agar was covered; the tubes were aseptically capped and stored at 5°C. These stock cultures were subcultured every 5 months.

EXAMINATION OF MORPHOLOGICAL CHARACTERS

Smears were made from colonies growing on 10% horse blood agar, or Blood Agar Base No.2, after an incubation period of 24 hours at 30°C, and stained by Gram's method. Smears were also made from nutrient broth cultures after incubation at 30°C for 24 hours, and these smears were stained with methylene blue. Observations on cell size, cell shape and cell arrangements of all the 406 strains were made on the methylene blue stained smears, since it was noticed that Gram's reagents, crystal violet and iodine, seemed to enlarge the size of the cocci in smears much more than the methylene blue, and therefore the latter stain allowed more accurate measurements of cells to be made. Cell diameters were measured with a micrometer eye-piece at a magnification of 1200 on individual cells, where this was possible. The measurement of the diameters of individual cells was straight forward for the majority of strains, but some strains exhibited packet and cluster formation with few individual cells. The latter group of strains invariably contained pleomorphic cells of a relatively large size and these were difficult to measure accurately. An average of five diameter

measurements of the cocci were taken in order to reduce the
measurements of the cocci were taken in order to reduce the
colonies growing on agar and the type of growth in broth culture were also noted.

EXAMINATION OF PHYSIOLOGICAL CHARACTERS

The optimum temperature of incubation

Streak cultures of all 406 strains were incubated on Blood Agar Base No.2 at 10°C, 25°C, 30°C and 40°C, and examined after 24 hours, 42 hours, 66 hours, 5 days, 9 days, 21 days, and finally 28 days' incubation. The temperature of the incubators did not fluctuate more than ± 1.0°C. Although many strains grew after 24 hours at 30°C or 40°C, some strains took more than two weeks to show any signs of growth at 10°C. The optimum temperature of incubation of strains was determined visually from the examination of the amount of growth appearing after 5 days at the above temperatures.

Pigmentation

The ability of all strains to produce pigments was examined on the following medium:- 10% (v/v) skim milk in Blood Agar Base No.2. Inoculated plates were incubated for 5 days at 30°C, and the shades of the pigments determined from the colonies without reference to colour charts.

Sodium chloride resistance

The ability of all strains to grow on varying concentrations of sodium chloride (NaCl) was examined on the following

basal medium:-

(% $\frac{W}{V}$ in distilled water) yeast extract, 0.3; peptone, 0.3; glucose, 0.1; agar, 1.5; sodium chloride impurity, 0.07; to this base was added various concentrations of sodium chloride ($\frac{W}{V}$), 5, 10 and 15. Streak cultures of all the strains were made on the basal medium containing 0, 5, 10 and 15% sodium chloride. The plates were incubated for 10 days at 30°C, and the cultures were examined for growth after 2, 6 and 10 days' incubation. Most strains showed growth after 2 days, but some strains took at least 6 days to show any visible signs of growth on 15% NaCl agar.

The few strains that grew on a concentration of 5 but not 10% sodium chloride in agar were plated on Mannitol Salt Agar (Oxoid), which contains a concentration of 7.5% sodium chloride, and the cultures were examined for growth after 2, 6 and 10 days at 30°C. Strains able to grow on both 5% and 10% sodium chloride agar were assumed to be able to grow on 7.5% Mannitol Salt Agar, especially as many of these strains were isolated on Mannitol Salt Agar (Table 23).

Catalase production

The ability of all strains to produce the enzyme catalase, was determined by adding 3% ($\frac{V}{V}$) hydrogen peroxide to colonies growing on Blood Agar Base No.2 after 5 days' incubation. Effervescence from the colonies, but not from the surrounding medium, was considered to be an indicator of catalase production.

Oxidase production

The ability of all strains to produce the enzyme oxidase was determined by adding 2 drops of the following reagent on to 4 day and 10 day old colonies growing on Blood Agar Base No.2 and on 10% NaCl agar medium (page 101) incubated at 30^oC.

Reagent:- 1%($\frac{W}{V}$) tetramethyl-p-phenylenediamine dihydro-chloride (D.D.H.) and 0.1%($\frac{W}{V}$) ascorbic acid in distilled water (Steel 1962b). A strong purple blue colour appearing within 30 seconds on the colonies, but not on the media, is characteristic of oxidase activity. The reagent auto-oxidises to the purple colour within a few days, despite the presence of the reducing agent, ascorbic acid, and therefore was always used immediately after its preparation.

Production of acid from carbohydrates

Glucose and mannitol

The ability of strains to produce acid from carbohydrates was examine in the following media:-

Glucose Agar Medium No.1 - (% $\frac{W}{V}$ in distilled water) $NH_4H_2PO_4$ 0.1; KCl, 0.02; $MgSO_4$ $7H_2O$, 0.02; yeast extract (Difco), 0.1; glucose, 1.0; bromocresol purple, 0.004; agar, 0.2; pH 7.0 (Baird-Parker, 1963).

Glucose Broth Medium No.1 - as Glucose Agar Medium No.1, but without any agar.

Glucose Agar Medium No.2:- (% $\frac{W}{V}$ in distilled water) tryptone

(Difco), 1.0; yeast extract (Difco), 0.1; glucose, 1.0; bromocresol purple, 0.004; agar, 0.2; pH 7.2 (Medium of Subcommittee on Taxonomy of Staphylococci and Micrococci, 1965b).

Glucose Broth Medium No.2 - as Glucose Agar Medium No.2 without any agar.

The following media were prepared in the same way as those listed above except that 1% mannitol was substituted for 1% glucose:-

Mannitol Agar Medium No.1   Mannitol Agar Medium No.2

Mannitol Broth Medium No.1   Mannitol Broth Medium No.2

The basal constituents of all the above media were sterilised at 10 lb./sq.in. for 20 minutes; 10% carbohydrate solutions, sterilised by Seitz filtration, were added aseptically to the sterile basal media to give a final concentration of carbohydrate in the media of 1%. The still-molten media were aseptically dispensed in 8 ml. amounts into sterile 6 x ⅝ in. test tubes which were then steamed for 15 minutes to drive off dissolved oxygen, and finally kept at 5°C until set (if the medium contained agar), or until cool (if there was no agar in the media).

Duplicate tubes were stab inoculated throughout their length with inocula from slope cultures. One tube was incubated under aerobic conditions to allow oxidation of the carbohydrate by the organisms to take place; the other tube

was incubated under anaerobic conditions to allow fermentation to take place.

Two methods of obtaining anaerobic conditions were used:-

(1) covering the surface of the inoculated media in the tube with 1-2 inches of sterile liquid paraffin,

(2) placing the tubes of inoculated media in an atmosphere of hydrogen in a modified all-metal McIntosh & Fildes anaerobic jar.

Utilisation of the carbohydrates was determined by acid production and a corresponding lowering of the pH which changed the colour of the indicator, bromocresol purple, from purple to orange or yellow in a pH of 6.8 or less.

All the inoculated media were examined for growth and acid production after 7, 14, 21 and 28 days' incubation at 30°C.

The growth and production of acid from mannitol by strains under aerobic conditions was also observed on Mannitol Salt Agar (page 101) after 5, 7, 14, 21 and 28 days' incubation at 30°C. Mannitol Salt Agar contains phenol red as a pH indicator with a pH range of 6.8 - 8.4, whereas all the other listed carbohydrate media contain bromocresol purple as a pH indicator with a pH range of 5.2 - 6.8; both pH indicators turn yellow in the acid pHs of their respective ranges.

<u>Lactose</u>

The ability of all strains to produce acid from lactose was examined in the following medium:- (% $\frac{W}{V}$ in distilled water)

lactose, 1.0; peptone, 1.5; Andrades indicator, 1.0. This medium, dispensed in 3 ml. amounts into 4 x ½ in. test tubes, was sterilised by steaming for 3 consecutive days. Tubes were examined for acid production, indicated by a pink colour in the medium, after 2, 4, 6 and 14 days' incubation at 30°C. Although the majority of strains which utilised lactose showed a positive reaction after 2 days, a few strains were late lactose utilisers and did not show any sign of acid production until a period of 14 days had elapsed.

β-galactosidase activity

Lowe (1962) discovered by means of the ONPG test that coliforms utilise lactose in 2 stages - first of all a permease is required to enable the lactose to penetrate the cells, and secondly a β-galactosidase is required to hydrolyse the lactose. Lowe (1962) also found by means of the ONPG test that some coliform strains do not have the ability to produce a permease, but apparently produce a β-galactosidase. A year later McClatchy & Rosenblum (1963) found, again by means of the ONPG test, that Staphylococcus aureus utilises lactose in the same way as the coliforms.

The ability of strains to utilise lactose without the requirement of a permease enzyme was examined in the following medium (Lapage & Jayaraman, 1964):-

0.6gm.O - nitrophenyl - β - D - galactopyranoside (ONPG) was

dissolved in 100 ml. of 0.01 M Na_2HPO_4 buffer at pH 7.5, at room temperature. This solution was sterilised by Seitz filtration and 1 part of ONPG solution was added aseptically to 3 parts 1% ($\frac{W}{V}$) peptone water at 7.5 pH, and dispensed in 3 ml. amounts in 4 x ½ in. test tubes. The yellow colour, due to the liberation of o - nitrophenol by the β-galactosidase enzyme, is characteristic of a positive reaction. Inoculated tubes were examined for positive reactions after 2, 3 and 7 days' incubation at 30°C.

Starch hydrolysis

The ability of all strains to hydrolyse starch was examined in the following medium:- 1% ($\frac{W}{V}$) soluble starch in Blood Agar Base No.2.

Petri dishes containing this medium, after inoculation and incubation at 30°C for 5 days, were flooded with Gram's iodine. The iodine reacted with the starch in the medium to give a blue black colour, and starch hydrolysis was detected by non-staining zones around the bacterial colonies.

Growth in glucose ammonium phosphate medium

Growth of strains of bacteria in Hucker's (1924a,b) prototroph medium is an indicator of the ability of those strains to utilise ammonium phosphate as sole source of nitrogen. This medium contains ($\frac{W}{V}$) 1.0% glucose, 0.1% ammonium phosphate, 0.02% potassium chloride, 0.02% magnesium sulphate, 0.004% bromocresol purple (pH indicator) and 1.5% washed agar in

distilled water. Hucker (1924a,b) used the agar medium in slopes, but I used the agar medium in 4 ml. deeps. The ability of the 406 strains to grow in this prototroph medium was examined in the following way:- for each strain, a small amount of colonial growth was emulsified in isotonic saline to give a faint opalescence. A needle point of this suspension was stabbed into a tube of the prototroph medium, which was then incubated at 25°C (the optimum temperature of prototrophic Gram positive cocci) for a period of 6 weeks. If growth occurred, a needle point of this growth was suspended in saline and another tube of prototroph medium was inoculated. This growth transfer procedure was repeated for a total of 6 times, or less if growth ceased to occur before the 6th transfer. If growth of a strain was recorded after the 6th and final transfer, the strain was considered to be prototrophic and able to utilise ammonium phosphate as a sole source of nitrogen.

<u>Acetoin production</u>

The ability of all strains to produce acetoin was examined in the following media:-

Acetoin Medium No.1 : Glucose phosphate medium (Mackie & McCartney, 1960; page 218).

Acetoin Medium No.2 : Unbuffered glucose broth (Baird-Parker, 1963).

Tubes containing 5 ml. of these media were inoculated and

incubated for 4 and 14 days. The presence of acetoin was detected by Barrits' (1936) method: 1.5 ml. of a 5% solution of α-napthol in absolute alcohol followed by 0.5 ml. of a 50% potassium hydroxide solution was added to 5 ml. of the broth cultures. A positive reaction, indicating the presence of acetoin, was recognised by a bright pink colour appearing at the surface of the culture after a time of 30 minutes to 3 hours.

Coagulase production

The ability of all strains to produce coagulase was examined by 3 methods of tube test.

Tube test 1

The type of plasma used in the test seems to be critical, but opinions vary as to which is the best. Fisk's method (1940), the tube test with human or rabbit plasma, seems to be the most widely accepted and was used by Mossel (1962), Pike (1962) and Baird-Parker (1963). Chapman *et al.* (1938) found that human plasma was much more effective than cow's plasma and that clots from human plasma lasted longer than clots from the rabbit plasma. Shaw *et al.* (1951) experimented with human, rabbit, horse and sheep plasma; the first two gave the greatest number of positive results. Smith (1962), working with strains of cocci isolated from dogs, noticed that a greater number of positive results were obtained from rabbit or sheep plasma than from human plasma. The most suitable plasma for the test may

well depend on the source of the strains to be examined. Since a large proportion of my strains were isolated from human skin, I used human plasma and tube test 1 was carried out.

Tube test 1 : 0.5 ml. of citrated fresh human plasma, diluted 1/10 in isotonic saline, was added to 0.5 ml. of an overnight nutrient broth culture of each strain in a tube. The tubes were examined for clot formation after 1, 4, 6 and 18 hours' incubation at 37°C.

Tube test 2

The standard temperature of incubation is 37°C, but Chapman et al. (1938) and Fisher (1936) obtained the same number of positive results at room temperature as at 37°C. Lominski et al. (1955) point out that a clot will occur at room temperature in plasma, provided that coagulase is preformed, before coagulase-destroying enzymes, which are known to be produced by staphylococci (Lominski et al. 1953) become active. Tube test 2, therefore, was carried out, in which the incubation temperature was lower and the plasma in a greater concentration, than in tube test 1.

Tube test 2 : 0.8 ml. of citrated fresh undiluted human plasma was added to 0.2 ml. of an overnight nutrient broth culture of each strain. These tubes were incubated at room temperature for 2 days and examined at intervals for clot formation.

Tube test 3

Although citrated plasma will only be clotted by staphylococci, some other bacteria will utilise the citrate, after an incubation period of 18-24 hours at 37°C, and allow the normal clotting mechanism to take place (Harper & Conway, 1948). The addition of heparin, which is active against thrombin in plasma, and inhibits the normal blood clotting action even in the presence of calcium ions, to citrated plasma was recommended by Lominski & Grossfield in 1948 to prevent the possibility of such false positive results. Tube test 3 was, therefore, carried out.

Tube test 3 : Citrated fresh human plasma containing heparin (10 international units/ml.) was substituted for the citrated fresh human plasma in tube test 2.

Slide test

The ability of all 406 strains to produce coagulase was also examined by the slide test. A slide test for the rapid identification of coagulase positive staphylococci was developed by Cadness-Graves et al., 1943 (page 53) in which a heavy suspension of staphylococci is mixed with a drop of plasma or fibrinogen solution on a microscope slide and if clumping occurs in under a minute then the staphylococcal strain is considered to be coagulase positive.

Slide test : A loopful of an overnight slope culture on Blood Agar Base No.2 was emulsified in a drop of saline or water to

which was added a loopful of undiluted citrated fresh human plasma. If no emulsification of the organism was possible, or if spontaneous granulation or clumping took place in saline or water alone, it was not possible to examine this strain by the slide coagulase test. A positive reaction was indicated by a clumping of the organism in the plasma in 15 to 60 seconds.

Phosphatase production

The ability of all strains to produce phosphatase was examined in the following medium:- 1 ml. of a 1% (w/v) Seitz filtered solution of sodium phenolphthalein diphosphate (L. Light & Co. Ltd.) in 99 ml. of Blood Agar Base No.2. Inoculated plates were incubated at 30°C for 5 days; the plates were then exposed to ammonia vapours and the phosphatase producing colonies were detected since they turned deep pink within 15 seconds. This pink colour is due to the reaction of ammonia with free phenolphthalein, released from phenolphthalein di-phosphate by the phosphatase enzyme.

Proteolysis

The ability of all strains to break down protein was examined by the following methods:-

(1) Strains were inoculated on to Blood Agar Base No.2 containing 10% (v/v) sterile skim milk, and incubated at 30°C for 5 days. The plates were then flooded with acidic mercuric

chloride (Frazier, 1926) to distinguish between true proteolysis and the clearing of milk, owing to the dissolving of milk protein by alkaline and products of metabolism (Hastings, 1904).

(2) Strains were inoculated on to Blood Agar Base No.2 containing 1% (w/v) gelatin and incubated for 5 days at 30°C. Gelatin hydrolysis was detected by the above mentioned method of Frazier (1926).

Urea hydrolysis

The ability of all strains to break down urea was examined in Christensen's (1946) medium. After the strains were incubated on this medium for 3 days at 30°C, the cultures were examined for their ability to hydrolyse urea, indicated by a purple colour developing in the medium.

Lipolysis

The ability of all strains to break down fat was examined on tributyrin agar and egg yolk agar as follows:-

(1) Strains were inoculated on to Tributyrin Agar (Oxoid) and incubated for 3 days at 30°C. Lipolysis was detected by clear areas developing around colonies on the opaque medium.

(2) Strains were inoculated on to Blood Agar Base No.2 containing 10% (v/v) egg yolk emulsion (Oxoid). Lipolysis was detected by areas of opalescence around colonies after an incubation period of 3 days at 30°C. Opalescence is thought

to be caused by a lipase (Gillespie & Alder, 1952).

Haemolysin production

The ability of strains to produce haemolysins was examined by the following methods:-

Haemolysin Method No.1 : 260 randomly chosen strains were streaked across plates of Blood Agar Base No.2 containing 10% ($^{v}/_{v}$) defibrinated horse blood (Oxoid). The plates were incubated aerobically at 30°C and observed for haemolysis after 24 and 72 hours. After 72 hours plates were left at 5°C overnight to detect the presence of "hot-cold" or β-haemolysin.

Haemolysin Method No.2 : This method differs from that of Lominski & Arbuthnott (1962) only in that an atmosphere of air was used instead of an atmosphere of 80% air and 20% carbon dioxide for the culturing of the strains.

A random selection of 18 of the strains, which were examined by Haemolysin Method No.1, were grown on sloppy agar plates, composed of 0.5% ($^{w}/_{v}$) agar in Nutrient Broth (Oxoid) for 60 hours. The plates were then deep-frozen and thawed. Following this procedure, the bacterial cells and agar were separated by low speed centrifugation from the supernatant containing the haemolysin; this latter portion was retained. Dilutions of $^{1}/1$, $^{1}/2$, $^{1}/4$, $^{1}/8$ were prepared from each supernatant in a diluent containing 75% saline, 24% Nutrient Broth and 1% of $^{1}/100$ dilution of thiomersalate. At this

stage, 2% suspensions of washed red blood cells of horse (Oxoid), human (expired blood, Western Infirmary, Glasgow), rabbit (Dept. of Bacteriology-Immunology, Glasgow University), mouse (Dept. of Bacteriology-Immunology, Glasgow University) and sheep (Oxoid) were prepared, and 0.5 ml. of each haemolysin dilution was added to 0.5 ml. of a red cell suspension and the mixture incubated for 6 hours. The titre of haemolytic activity was calculated from the highest dilution of haemolysin which would lyse 50% of the red cells after this time.

Phage typing

A random selection of 89 strains were phage typed at the phage typing laboratory, Department of Bacteriology-Immunology, Western Infirmary, Glasgow. The international standard method was used in which *Staphylococcus aureus* phages of Groups I, II, III and the miscellaneous group (sometimes called Group IV) were tested on each of the 89 strains. The phages were supplied by the Staphylococcus Reference Laboratory, Colindale Avenue, London, N.W.9.

PHOTOGRAPHY

Diagrams, Tables and Scheme 1

The diagrams, some of the tables, and Scheme 1 were photographed with a half plate or quarter plate M.P.P. camera on to Kodalith Ortho film (Kodak), and were printed on Kodagraph Projection paper (Kodak) or bromide air mail paper (Kodak).

SECTION A

EXAMINATION OF THE MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERS OF 406 STRAINS OF MICROCOCCI AND STAPHYLOCOCCI

RESULTS

RESULTS

INTRODUCTION TO THE RESULTS' SECTION

In dealing with the morphological and physiological results of the 406 strains, I have used tables showing a simple arbitrary classification scheme of the strains into 4 groups, on thebasis of glucose utilisation and coagulase production.

Strains were first of all divided into 2 main groups - staphylococci and micrococci - by the character of glucose fermentation; glucose fermentation is generally recognised as the sole character which separates staphylococci from micrococci (page 14).

The staphylococci were divided further into 2 groups - coagulase positive staphylococci and coagulase negative staphylococci; coagulase production is regarded as the main character separating the pathogenic from the saprophytic staphylococci (page 54).

The micrococci were further divided into 2 groups - the glucose oxidising micrococci andthe micrococci unable to utilise glucose; the group, containing strains unable to utilise glucose even under aerobic conditions, comprises most of the pigmented strains of the Micrococcaceae, e.g. strains of Micrococcus luteus and M. roseus.

These groups are presented with a view to help the reader compare my results with those of other taxonomists, who have used the characters of glucose utilisation and coagulase production as main characters in their classification schemes.

(Tables 2, 3, 4 and 5); I do not recognise such simple groups, however, in my classification schemes (Schemes 1 & 2).

MORPHOLOGICAL RESULTS

Cell size and cell shape

The diameters of cells of all 406 strains were measured, and it was found that diameters ranged from 0.9μ to 1.5μ; the results are summarised in Table 24.

Five out of the 406 strains contained cells which were ellipsoidal in shape, and it was difficult to tell if these strains were really cocci or short rods. These strains, however, were classified with the others, for if they were different physiologically as well as morphologically, the Adansonian method of classification would automatically place them in a separate group from the truly spherical cocci, (page 89).

Gram's reaction of cells in stained smears

All 406 strains were found to be Gram positive when they were stained by Gram's method.

Cell arrangement, colonial appearance of cells on agar and the form of growth in broth culture

Only randomly sampled strains were examined for cell arrangement, the form of colonies on agar and the type of growth in broth cultures. I found that no strain, including named *Micrococcus luteus* and *Sarcina* species, consistently showed packet formation, and like many other taxonomists, I do not

consider that packet formation is a significant character for classifying strains of micrococci and staphylococci.

The results are summarised in Table 24.

Table 24

Cell size, cell arrangement, colonial appearance and growth in broth of 406 strains of micrococci and staphylococci

Morphological appearances	coagulase +ve staphylococci	coagulase -ve staphylococci	non-packet forming micrococci	packet forming micrococci
Cell size (dia. in μ)	0.9	1.0	1.0 - 1.2	1.1 - 1.5
Cell arrangement	single cells, pairs, short chains and grape-like clusters.			packets of cells.
Colonial appearance	small to medium size colonies (0.5 - 2.0 mm.), entire and slightly convex.		medium to large size colonies (1.0 - 4.0 mm.), usually entire and convex yellow colonies often granular.	
Growth in broth	fine easily suspendible sediment.		non-pigmented strains have fine suspendible sediment; red and yellow pigmented strains have mucoid sediment with clear supernatant.	

Motility of cells

In view of the extreme rarity of motile forms of cocci (page 31), I did not examine any of the 406 strains for the character of motility.

The optimum and critical temperatures for growth

It was considered important to establish the optimum

temperature of growth and the critical growth range of all strains early on in the taxonomic studies of micrococci and staphylococci, so that the later experimental work would not be hindered by lack of knowledge of the temperature range of growth of the strains.

All strains grew at 25°C and 30°C, and the numbers of strains growing at 10°C and 40°C are listed in Table 25.

Table 25

Growth of the micrococcal and staphylococcal strains at 10°C and 40°C.

Arbitrary grouping of strains	No. of strains examined	Strains growing at	
		10°C	40°C
Coagulase +ve staphylococci	21	20	21
Coagulase -ve staphylococci	207	24	204
Glucose oxidising micrococci	130	118	78
Micrococci unable to utilise glucose.	48	36	22

As can be seen from the table, the coagulase positive strains grew at 10°C and 40°C, but most coagulase negative staphylococci grew at 40°C and not at 10°C; the majority of the micrococci unable to utilise glucose grew at 10°C but only 48% of these strains grew at 40°C.

Pigmentation

The shades of the pigments of the 406 strains were determined from a visual range of 14 shades of colour.

One strain, Micrococcus violagabriollae, strain no. 336, known to produce a violet pigment, did not do so on 10% skim milk agar, but the pigment was produced by the strain on tributyrin agar and starch agar. Since all the other strains were grown on these 3 media, I would have noticed any other strain producing a similar violet pigment. Apart from the violet pigment, all other pigments were well developed on colonies growing on 10% skim milk agar.

Of the 21 coagulase positive staphylococci, 13 strains produced pigments of varying shades of yellow, 6 strains were white, and 2 strains were cream coloured. Most of the coagulase negative staphylococci (175/207 strains) produced white colonies. The following strains were yellow pigmented: 12/207 coagulase negative staphylococci, 20/130 glucose oxidising micrococci and 26 (including all 13 culture collection strains of Micrococcus luteus) of 48 micrococci unable to utilise glucose. All the 10 strains of Micrococcus roseus produced a rose red pigment noticeably different from the deep red pigment of Micrococcus radiodurans (strain no. 339). Strain no. 333, Staphylococcus flavocyaneus produced a black diffusible pigment, in addition to a non-diffusible yellow pigment similar to that of other yellow pigmented micrococci and staphylococci.

Growth on various concentrations of sodium chloride in agar

Strains could be divided up into groups on the basis of

tolerance to various salt concentrations (page 35):
(a) 2% of the strains grew only on 0% and 5% NaCl agar (non-halotolerant), (b) 12% of the strains grew only on salt agar up to a concentration of 7.5% NaCl (non-halotolerant), (c) 25% grew on salt agar up to a concentration of 10% NaCl (non-halotolerant), and (d) 61% grew on salt agar up to a concentration of 15% NaCl (halotolerant). All 406 strains grew on 0% and 5% NaCl agar, and hence no halophilic strains were encountered.

The results of growth of strains on various concentrations of sodium chloride in agar are summarised in Table 26.

Table 26

Growth response of the micrococcal and staphylococcal strains on sodium chloride agar

Arbitrary grouping of strains	No. of strains examined	Limiting concentration of NaCl in agar for growth			
		5%	7.5%	10%	15%
Coagulase +ve staphylococci	(21)	*0	1	19	1
Coagulase -ve staphylococci	(206)	2	9	46	149
Glucose oxidising micrococci	(129)	0	11	25	93
Micrococci unable to utilise glucose	(47)	6	25	12	4

* No. of strains

It can be seen from the table that 10% sodium chloride was the limiting concentration for the growth of 19/21 coagulase positive staphylococci. Most of the coagulase negative

staphylococci and the glucose oxidising micrococci were halotolerant, but no yellow or red pigmented micrococci unable to utilise glucose grew on 15% NaCl agar, and the 4 strains able to grow on this medium produced white colonies.

Catalase production

Although bacterial taxonomists define micrococci and staphylococci as being catalase positive, I found 3 catalase negative strains which shared physiological characters with some of the catalase positive strains, and therefore I have classified these with the other 403 catalase positive strains (Scheme 1).

The catalase reaction of all the 406 strains growing in agar is compared with the presence of catalase bands (blood bands) detected in the cell contents of the same strains, which have been electrophoresed across acrylamide gels (page 216).

Oxidase production

I found that oxidase positive strains produced a deeper colour reaction with the oxidase reagent, when the cultures had been incubated for 10 days instead of 4 days. It would appear, therefore, that a long incubation period is required for the maximum production of oxidase. I also found that a greater number of oxidase positive results were recorded from strains grown on Blood Agar Base No.2 than on the same medium containing 10% sodium chloride; presumably the sodium

chloride inhibits the production of the oxidase enzyme in some strains. The procedure that I chose, therefore, for these tests for oxidase production was to plate out the strains on Blood Agar Base No.2, incubate for 10 days, and add the reagent to the colonies. The results are summarised in Table 27.

Table 27

Oxidase production by the micrococcal and staphylococcal strains

Arbitrary grouping of strains	No. of strains examined	Strains producing oxidase
Coagulase +ve staphylococci	21	0
Coagulase -ve staphylococci	207	4
Glucose oxidising micrococci	130	5
Micrococci unable to utilise glucose	47	38

As can be seen from the table, no coagulase positive staphylococcus, and very few coagulase negative staphylococci and glucose oxidising micrococci produced oxidase. The largest number of oxidase producing strains were the micrococci unable to oxidise glucose, and 30/38 of these oxidase positive strains were either yellow or red pigmented. These results are in agreement with those of Steel (1962b) (page 48), even though he did not study such a large number of strains as I did. Steel (1962b) found that no coagulase positive coccus produced an oxidase reaction, and strains showing oxidase activity

generally appeared to be oxidisers rather than fermenters of glucose.

Utilisation of carbohydrates

From the results of previous classification schemes, only a few carbohydrates appear to be useful in the classification of micrococci and staphylococci (page 42), and accordingly I used only 3 carbohydrates - glucose, mannitol and lactose - for observing oxidation and fermentation reactions, and starch in a solid medium for the observation of hydrolysis, by the 406 strains.

The production of acid from glucose and mannitol

There has been much controversy in recent years concerning the significance of "sugar" reactions in the classification of micrococci and staphylococci (page 39). Fermentation of glucose has generally been recognised as the sole character which separates the genus *Staphylococcus* from the genus *Micrococcus*. In addition fermentation of mannitol is a character typical of coagulase positive staphylococci. Since fermentation of glucose and mannitol are important characters in the classification of micrococci and staphylococci, I, first of all, had to find out (a) the best means of achieving anaerobic conditions, and (b) the most suitable media for the demonstration of acid production from glucose and mannitol under both aerobic and anaerobic conditions.

(a) Anaerobic conditions:

I found no difference in the amount of acid produced by 15

strains growing under aerobic and anaerobic conditions, in Glucose Agar Medium No.1 (page 102) and Mannitol Agar Medium No.1 (page 103), when liquid paraffin was used as a means of achieving anaerobiosis (page 104). When one set of duplicate tubes of the same media, inoculated with the same strains, however, was incubated in a McIntosh and Fildes anaerobic jar and the other set incubated under aerobic conditions, I found that some of the strains which produced acid under aerobic conditions, in both the glucose and mannitol agar media, failed to do so in the tubes incubated in the anaerobic jar. I was certain, therefore, that cultures incubated in an anaerobic jar were incubated under completely anaerobic conditions, whereas it seemed likely that the liquid paraffin was not truly effective in creating an anaerobic seal on the agar deeps. I consider that liquid paraffin has too many variables for its use as an anaerobic seal, e.g. the amount of air trapped at the interface of the paraffin and the media, the depth of paraffin cover, and the possibility of contamination of the media by airborne bacteria during layering. Most taxonomists, however, still use liquid paraffin as a means of achieving anaerobiosis for examining the fermentative ability of bacteria.

(b) Media:

The selection of the most suitable media for the demonstration of acid production, under both aerobic and anaerobic

conditions, was a more difficult problem to solve. First of all, there was the difficulty in deciding whether to use agar or broth media. The majority of the 406 strains either produced large amounts of acid, or apparently none at all, in both the agar media (Glucose and Mannitol Agar Media No.1), and the broth media (Glucose and Mannitol Broth Media No.1)(page 102), and the recording of these results was straight forward. There were, however, some strains, which seemed to produce very little acid either aerobically or anaerobically, since there was no clear colour change in the pH indicator. Occasionally, broth media were turned from a purple colour to a purple brown colour, and it was sometimes not easy to tell whether any acid had been produced or not. Agar media were either changed to the same purple brown colour or showed a distinct narrow zone of yellow at the top of the tubes over a broad purple zone at the bottom; this latter appearance only occurred in a few strains when they were incubated under aerobic conditions, but it was obvious that at least a little acid had been produced by these strains and for this reason agar media was preferred to the broth media for demonstrating acid production.

The length of the incubation period, however, is important if those strains producing little acid are to be recorded as weakly positive and not as negative, since I have observed slow but continuous pH changes in inoculated Glucose Agar Medium No.1, incubated for a period of 2 months. I noticed that 3 strains

growing aerobically in glucose agar deeps produced a narrow zone of yellow at the top of the agar after a few days, and this colour gradually spread throughout the whole of the medium, taking about 14 days to do so. After 28 days' incubation, the yellow colour had diminished to a broad zone at the bottom of the tubes; after 49 days' incubation all the medium in the tubes had reverted back to the original purple colour, and, unless the tubes had been examined regularly, these 3 strains would have been regarded as having no effect on the carbohydrate. The mechanism of the reaction was not investigated, but it seemed that after some acid had been produced from the carbohydrate, ammonia or other alkaline metabolic products raised the pH back to neutral or above. A few other strains were very slow in producing acid, either aerobically or anaerobically, from glucose and mannitol agar media, and whereas a tube of inoculated medium could be negative after 14 days' incubation, it might be weakly positive at 21 days' incubation. Therefore, it is important that inoculated media should be incubated for at least a month and looked at regularly during that time.

While these experiments were being carried out, the Sub-committee on Taxonomy of Staphylococci and Micrococci (1965b) proposed Glucose Agar Medium No.2 (page 102) as the standard medium for the demonstration of fermentation of glucose by staphylococci, and the Subcommittee recommended that this

medium should be used for the separation of the genera *Staphylococcus* and *Micrococcus*. Accordingly, I used this medium in both the agar and broth forms for the demonstration of acid production from glucose (Glucose Agar and Broth Media No.2, page 102). I substituted mannitol for glucose in the same basal medium, and I used Mannitol Agar and Broth Media No.2 (page 103) for the demonstration of acid production from mannitol. I compared the results obtained from the new media with those of other media.

Glucose fermentation and oxidation

I compared the activity of 93 strains inoculated into both Glucose Agar Medium No.1 and Glucose Agar Medium No.2, and a greater number of glucose fermenting strains were detected in the latter medium. Glucose Agar Medium No.1 : 82 strains fermented glucose, 7 strains oxidised glucose and 1 strain did not attack glucose at all; finally 3 strains gave reactions impossible to interpret.

Glucose Agar Medium No.2 : 82 strains fermented glucose, 6 strains oxidised glucose and 1 strain did not attack glucose at all. Therefore, the medium I adopted for the demonstration of acid production from glucose under both aerobic and anaerobic conditions for all 406 strains was Glucose Agar Medium No.2. Cultures were examined at weekly intervals for 28 days. If any transient zones of yellow appeared in the agar medium during the incubation period,

the strains giving such a reaction were considered to be weakly positive.

In my examination of the 406 strains for glucose fermentation and oxidation in Glucose Agar Medium No.2, I found that 228 (57%) strains fermented glucose, 130 (31%) strains could not ferment but oxidised glucose, and 48 (12%) were unable to utilise glucose at all. All the 21 strains of coagulase positive staphylococci were glucose fermenters.

Mannitol fermentation and oxidation

Mannitol fermentation: When 100 strains were examined for fermentation of mannitol in Mannitol Agar Medium No.1 (page 103) and Mannitol Agar Medium No.2 (page 103), 17 strains were recorded as mannitol fermenters with both media.

Mannitol oxidation: Table 28 shows the comparative results for acid production in four mannitol media under aerobic conditions by 7 coagulase negative micrococci and staphylococci after one month's incubation at $30^{\circ}C$.

Table 28

Mannitol oxidation results of 7 strains of micrococci and staphylococci on 4 mannitol media

Strain No. & genus	Media dispensed in			
	petri dishes	tubes		
	Mannitol Salt Agar	Mannitol Agar Medium No.1	Mannitol Broth Medium No.2	Mannitol Agar Medium No.2
65 M	+W	+	-	+W
82 M	+	+	+W	+W
83 M	+	+	+	+
91 M	-	-	+	+
110 S	+	+	+	+
133 S	+ after 5days then -	+	-	+W
139 M	+ after 5days then -	+	+W	-

S = *Staphylococcus*:- able to ferment glucose in Glucose Agar Medium No.2.

M = *Micrococcus*:- unable to ferment glucose in Glucose Agar Medium No.2.

+ = strong production of acid   +W = weak production of acid
- = no production of detectable acid.

The complex nature of the results of strains 133 and 139 on Mannitol Salt Agar are difficult to explain, and these results merely reflect the reactions of many metabolic pathways active on the constituents of this medium (page 96): it was assumed that even if a transient positive reaction, as far as acid production was concerned, took place, then the strains would still be recorded as a mannitol oxidiser. Only 4 (3 micrococci and 1 staphylococcus) of the 7 strains produced acid in all 4 media, the remaining strains being positive only in some of the media. Although these media are complex and should allow any strain capable of utilising sugars to produce acid, these results reflect slight but significant constituent differences, as far as the amount of acid produced required to change the colours of the pH indicator is concerned. Mannitol Salt Agar differs from the other media in most of its constituents (page 96), but the only differences between Mannitol Agar Medium No.1 and Mannitol Agar Medium No.2 are that the former medium includes 3 salts among its constituents and the latter medium contains tryptone (pages 102 & 103). It is interesting to note that the only difference between Mannitol

Agar Medium No.2 and Mannitol Broth Medium No.2 is that the former contains agar and the latter does not (page 103), yet two strains were recorded as weakly positive (nos. 65 and 133) in the Agar and negative in the Broth.

Using a larger number of strains, I compared the results of acid production from mannitol under aerobic conditions in both Mannitol Salt Agar and Mannitol Agar Medium No.2. There was a 94% (328/349 strains) correlation between the results in the 2 media; the remaining 6% of the strains gave the following results:- (a) 2% did not grow on the Mannitol Salt Agar, because of its 7.5% sodium chloride concentration, (b) 3% produced acid in Mannitol Agar Medium No.2 but not in Mannitol Salt Agar, and (c) 1% produced acid in Mannitol Salt Agar but not in Mannitol Agar Medium No.2.

In these experiments I have shown that more strains produce acid from mannitol under aerobic conditions when they are grown in Mannitol Agar Medium No.2 than in any other of the media tested.

In summarising the results of the experiments with the mannitol media, I found that (a) strains fermented mannitol identically in both Mannitol Agar Media Nos. 1 and 2. (b) Mannitol Agar Medium No.2 was the best of all the mannitol media examined for detecting mannitol oxidising strains. Consequently, the medium of choice for the detection of fermentation and oxidation by strains of micrococci and

staphylococci was Mannitol Agar Medium No.2.

Cultures were examined at weekly intervals for 28 days. If any transient zones of yellow appeared in the agar medium during the incubation period, the strains giving such a reaction were considered to be weakly positive.

In my examination of the 406 strains for mannitol fermentation and oxidation in Mannitol Agar Medium No.2, I found that 31 strains (8%), including 19/21 coagulase positive staphylococci, fermented mannitol, 126 strains (31%) did not ferment but oxidised mannitol, and 149 strains (61%) were unable to utilise mannitol at all.

The viability of strains in Glucose and Mannitol Agar Media No.2 during the incubation period

I considered it was important to find out whether some strains might die off during the 28 day incubation period in these media, before producing any visible colour change in the pH indicator, and, therefore, give rise to negative reactions, owing to the death of the cells, rather than the strains' inability to produce the appropriate enzymes. Accordingly, loopfuls of 28 day old aerobic and anaerobic cultures of a variety of strains in Glucose and Mannitol Agar Media No.2 were plated out on to Blood Agar Base No.2, and the plates incubated aerobically at 30°C. Most strains, which produce acid to give a rapidly formed yellow colour throughout the length of the tube, die off owing to the detrimental effect

of the low pH; strains which do not produce acid, or do so very weakly, remain viable in Glucose Agar Medium No.2 under aerobic and anaerobic conditions, and also in Mannitol Agar Medium No.2 under aerobic conditions. Only when strains were incubated in Mannitol Agar Medium No.2 anaerobically and did not ferment the sugar, were many of them unable to survive the 30 day incubation period; the presence of mannitol under anaerobic conditions must be inhibitory in some way to at least some of the strains. It is interesting to note that I found that strains either fermented mannitol with a rapid colour change in the medium, or did not ferment the sugar at all with no colour change - there were no weakly positive strains: this may be accounted for by the fact that strains can survive in mannitol-containing media under anaerobic conditions for a limited period of time, and there is no opportunity for slowly fermenting strains to metabolise enough sugar to give weakly positive reactions. It would seem, therefore, that negative reactions are recorded for glucose fermentation and oxidation, and mannitol oxidation, when strains grow in the sugar media, but fail to produce any visible amounts of acid; and negative reactions are recorded for mannitol fermentation in Mannitol Agar Medium No.2 when strains grow but fail to produce any acid, or when strains are unable to grow. The fact that negative reactions in sugar media can be due to 2 reasons, supports the argument that

fermentation and oxidation of "sugars" should not be used as main characters in a classification scheme of micrococci and staphylococci (page 43).

Combined results of glucose and mannitol utilisation

Of the 406 strains, 31 (8%) fermented both glucose and mannitol, 43 (11%) fermented glucose and oxidised mannitol, 154 (38%) fermented glucose but were unable to utilise mannitol at all, 83 (20%) oxidised both glucose and mannitol, 47 (11%) oxidised glucose but were unable to utilise mannitol at all, and 48 (12%) were unable to utilise glucose or mannitol at all.

It was found that invariably if strains fermented mannitol they were able to ferment glucose and oxidise both glucose and mannitol, if strains fermented only glucose, they were able to oxidise glucose, and if strains oxidised mannitol they were able to oxidise glucose. These observations indicate that glucose is metabolised along part of the mannitol metabolic pathway, but further work is required to elucidate the complete relationship between the mannitol and the glucose metabolic pathways.

Lactose utilisation and the ONPG test

All 406 strains were examined for acid production from lactose in peptone water under aerobic conditions (page 104); 281 strains (including all 21 coagulase positive staphylococci)

produced acid from lactose within 2 days, 6 strains produced acid from lactose only after 10-14 days, and 119 strains did not produce any acid from lactose even after 14 days' incubation. It was thought that these late lactose utilisers might lack a permease system or have a weak one, since it has been shown that Staphylococcus aureus utilises lactose in two stages, involving a permease and a β-galactosidase (page 105).

I grew 10 lactose utilising strains (including the late lactose utilising strains) in ONPG medium and all 10 strains were found to produce the yellow colour of o-nitrophenol. From these results, I assumed that strains producing acid from lactose would also produce o-nitrophenol from ONPG, and I did not examine any other strains producing acid from lactose by the ONPG test. All 119 strains failing to produce acid from lactose, however, were grown in ONPG medium, and 30 of these strains hydrolysed the ONPG. The results of the hydrolysis of lactose and ONPG by the strains are summarised in Table 29. It can be seen from this table that only in the coagulase positive staphylococcus group are all the strains capable of producing acid from lactose, and each of the other groups - coagulase negative staphylococci, glucose oxidising micrococci, and micrococci unable to utilise glucose - contains strains capable of producing o-nitrophenol from ONPG, but incapable of producing acid from lactose.

These results probably indicate that the latter strains, which hydrolyse a β-galactopyranoside, but not lactose, lack a permease system, and it would appear that the micrococci and staphylococci which utilise lactose do so in 2 stages, one stage involving a permease, and the other stage involving a β-galactosidase. The conclusion of McClatchy & Rosenblum (1963), who showed that Staphylococcus aureus utilises lactose in the same 2 stages, therefore, has been extended by my results to include all micrococci and staphylococci.

From a taxonomic point of view, the results of lactose and ONPG hydrolysis do not correlate particularly well with other characters, although, in general terms, the ability of a strain to produce acid from lactose is proportional to the strain's ability to ferment mannitol and glucose.

It is interesting to note that 4 strains unable to utilise the monosaccharide glucose should be able to utilise the disaccharide lactose. This observation is commented further on page 164 .

Table 29

The aerobic breakdown of lactose and ONPG by the micrococcal and staphylococcal strains

Arbitrary grouping of strains	No. of strains examined	Strains hydrolysing lactose +	lactose -	ONPG (lactose -ve strains) +	ONPG (lactose -ve strains) -
Coagulase +ve staphylococci	21	21	0	2 lactose +ves are ONPG +ve	
Coagulase -ve staphylococci	207	178	29	5	24
Glucose oxidising micrococci	130	84	46	15	31
Micrococci unable to oxidise glucose	48	4	44	10	34

\+ = positive reaction - = negative reaction

Starch hydrolysis

All 406 strains were examined for their ability to hydrolyse starch in starch agar. Only 46 strains hydrolysed starch - 11/228 (5%) glucose fermenting staphylococci (including 2 coagulase positive strains), 3/130 (2%) glucose oxidising micrococci, and 33/48 (69%) micrococci unable to utilise glucose. The group containing the largest number of strains capable of hydrolysing starch in agar also contains the most biochemically inactive of the micrococci, e.g. strains of Micrococcus luteus and M. roseus and it is interesting to note that these strains, which hydrolyse starch presumably eventually to glucose, could not produce acid from glucose in the complex Glucose Agar Medium No.2; this is discussed further on page 164.

Growth in glucose ammonium phosphate medium

Of the 406 strains tested in glucose ammonium phosphate medium, only 16 (4%) strains survived 6 serial transfers through the agar medium; a further 6 strains grew on the agar after 3 serial transfers, but failed to grow on the fourth, and I considered these 6 strains to be wekly prototrophic. All 22 strains were micrococci and 15 of the 16 strong prototrophs were yellow pigmented micrococci unable to utilise glucose. I found a much lower percentage of Gram positive, catalase positive cocci to be prototrophic in comparison with the findings of other taxonomists (page 36). This is probably because I used 6 serial transfers through glucose ammonium phosphate medium to ensure that there was no carry over of nutrients, whereas no other taxonomist used a transfer technique, but judged prototroph activity on the result of one inoculation, and undoubtedly some of their "prototrophs" grew on the nutrients carried over with the inoculum.

Although I used the character of growth in glucose ammonium phosphate medium as a sole indicator of prototroph activity, according to Hucker (1924a,b), acid production with a change of colour should also occur with growth, since glucose is the only source of energy the organisms have, and the end products of glucose metabolism are acids. I found that only 9 of the 16 strongly prototrophic strains produced demonstrable amounts of acid. The possible reasons for this finding are discussed on page 165 .

Acetoin production

Before all the 406 strains were tested for their ability to produce acetoin, a suitable incubation period was determined for a limited number of strains. Two batches of both Acetoin Medium No.1 (page 107) and Acetoin Medium No.2 (page 107) were inoculated with 17 randomly chosen strains of micrococci and staphylococci. One batch was examined for acetoin production after 4 days and the other batch after 14 days' incubation - 6 acetoin producing strains were recorded after 4 days, and 7 after 14 days. It seems that at least 14 days' incubation is required to detect all the late acetoin producers, and apparently no break-down of acetoin, already formed, takes place during this incubation period; these results disagree with those of Eddy (1961) but agree with those of Baird-Parker (1963) (page 44). The 406 strains, therefore, were incubated in Acetoin Medium No.1 and No.2 for 14 days before testing for the presence of acetoin.

Two media, Acetoin Medium No.1 and No.2, were used in the detection of the presence of acetoin, since both media have been employed in micrococcal and staphylococcal classification schemes (Abd-el-Malek & Gibson, 1948; Shaw et al., 1951; Baird-Parker, 1963). Of the 406 strains cultured in both media, 221 strains were acetoin positive in Acetoin Medium No.1 and No.2, 10 strains were acetoin positive only in

Acetoin Medium No.1, and 18 strains were acetoin positive only in Acetoin Medium No.2. It would appear that the phosphate free medium (Acetoin Medium No.2) stimulates the production of acetoin by a few more strains than the glucose phosphate medium (Acetoin Medium No.1), but I do not attempt to explain the discrepancies of the results between the 2 media. Similar results have been recorded by the above authors who used both media, but no reason was ever given to explain the varying results in the 2 media. In fact, we know very little about the mechanism of acetoin production by both staphylococci and micrococci. This production seems to depend not only on the potentiality of the strains to produce acetoin, but also on the composition of the medium used, and this confirms my view expressed on page 45, that acetoin production should not be regarded as a main character in any classification scheme of these organisms until an internationally recognised standard method, which is capable of detecting all potential acetoin producers, is proposed. Therefore, instead of choosing the results from only one of the acetoin media, as other taxonomists have done, I have used both sets of results for classifying the 406 strains, and the results are summarised in Table 30.

All the coagulase positive staphylococci are acetoin positive, and the strains' ability to produce acetoin is approximately proportional to their ability to utilise

carbohydrates; however, it is surprising that at least some of the micrococcal strains which oxidise glucose (26/130 strains) or do not utilise glucose at all (4/48 strains), or apparently not in Glucose Agar Medium No.2, can produce acetoin, because the normal mechanism of acetoin production is dependent on pyruvic acid, arising from fermentative pathways starting from glucose. The significance of these results is discussed further on page 167.

Table 30

Acetoin production by the micrococcal and staphylococcal strains

Arbitrary grouping of strains	No. of strains examined	Strains producing acetoin		
		strong +ve	weak +ve	negative
Coagulase +ve staphylococci	21	20	1	0
Coagulase -ve staphylococci	207	163	9	35
Glucose oxidising micrococci	130	26	12	92
Micrococci unable to oxidise glucose	48	4	2	42

strong positive = strains which give acetoin +ve reactions in both media.

weak positive = strains which give acetoin +ve reactions in one medium but not the other, or strains which produce acetoin weakly in both media.

negative = strains which do not produce acetoin in either media.

Coagulase production

Tube test

Tube Test No.1 (page 108) is the one used routinely in most laboratories for the detection of coagulase positive strains. Tube Test No.2 (page 109) allows weak coagulase positive strains to produce a visible clot before any coagulase destroying enzyme can become active (page 109). Tube Test No.3 (page 110) includes the use of heparin in case any citrate utilising organism allowed the normal plasma clotting mechanism to take place (page 110).

Of the 406 strains, 21 were recorded as coagulase positive staphylococci by Tube Tests 1, 2 and 3. Two of these coagulase positive strains did not ferment or oxidise mannitol - the other 19 strains fermented both glucose and mannitol. However, 9/31 mannitol fermenting strains were coagulase negative; the reports in the literature, that the possession of the character of mannitol fermentation is closely associated with the possession of the character of coagulase production in staphylococci (page 42), is not supported by these findings.

Slide Test

Despite the fact that overnight cultures were used to examine strains by the slide test for "bound" coagulase, the results were disappointing in that they did not show a close correlation with those of the tube tests:- 27 slide coagulase

positive strains were recorded, of which only 9 were tube test coagulase positive; 64 strains autoagglutinated in saline and in water and could not be tested; 5 strains could not be emulsified in either saline or water and could not be tested.

It is possible that for a close correlation between slide test and tube test results can be found only when the strains tested are newly isolated, as they are in bacteriological routine testing laboratories in hospitals where these coagulase tests are most frequently used - most of the strains that I tested had been cultured for 2 years. I did not use these results for classifying the 406 strains.

Phosphatase production

Of the 406 strains tested, 274 strains were phosphatase positive, the reactions varying from strong to weak. All 21 strains of coagulase positive staphylococci, 73% of the coagulase negative staphylococci, 76% of the glucose oxidising micrococci, and 30% of the micrococci unable to utilise glucose were phosphatase positive. The results are summarised in Table 31. Phosphatase production is a character shown by many strains of micrococci and staphylococci, but it cannot be correlated with any other character demonstrated by these organisms.

Table 31

Phosphatase production by the micrococcal and staphylococcal strains

Arbitrary grouping of strains	No. of strains examined	Strains producing phosphatase
Coagulase +ve staphylococci	21	21
Coagulase -ve staphylococci	207	150
Glucose oxidising micrococci	130	99
Micrococci unable to oxidise glucose	47	14

Milk clearing and hydrolysis

All 406 strains were examined for their ability to break down casein in milk agar (page 111). Clearing of the milk was visible around colonies of some of the strains after incubation, but this clearing disappeared in many of the strains when acidic mercuric chloride was added to the plates to precipitate the casein. Therefore, it was possible to distinguish between clearing, which was caused by the dissolving of the milk protein by the alkaline byproducts of the cells, and true proteolysis, due to the hydrolysis of the casein (page 49). Of the 406 strains, 263 cleared milk agar and 169 hydrolysed casein in the milk agar; no strains were found that could hydrolyse casein without clearing milk agar. The milk hydrolysis results are summarised in Table 32. There appears to be no correlation between milk hydrolysis and any other character.

Gelatin hydrolysis

All 406 strains were examined for their ability to break down gelatin in gelatin agar (page 112), and of these, 182 strains were gelatin positive. The results are summarised in Table 32. There appears to be no correlation between gelatin hydrolysis and any other character.

Urea hydrolysis

All 406 strains were examined for their ability to hydrolyse urea in urea agar slopes (page 112), and 380 strains were found to hydrolyse urea to ammonia. The results are summarised in Table 32. There appears to be no correlation between urea hydrolysis and any other character.

Table 32

Casein, gelatin and urea hydrolysis by the micrococcal and staphylococcal strains

Arbitrary grouping of strains	No. of strains examined	Strains hydrolysing		
		Casein	Gelatin	Urea
Coagulase +ve staphylococci	21	10	17	20
Coagulase -ve staphylococci	207	123	98	157
Glucose oxidising micrococci	130	25	43	82
Micrococci unable to oxidise glucose	48	11	23	20

Tributyrin hydrolysis

Break-down of tributyrin was detected in Tributyrin Agar for all but 14 of the 406 strains. The results are summarised in Table 33.

Egg yolk opacity

I found that among the 406 strains, 100 biochemically active staphylococci were capable of causing opacity in egg yolk agar. Only 50, however, of these 100 strains showed opacity around all or most of the colonies; in the other 50 strains, only a few of the colonies on each plate were surrounded by a zone of opacity.

It was noticed that if a colony, surrounded by an opaque zone (positive), and a colony without such a zone (negative), were selected from a strain that produced a mixture of positive and negative colonies on egg yolk agar, and each of these colonies was plated out on to the same medium, the resultant colonies were, in each case, again a mixture of positive and negative egg yolk opacity colonies. This result was not expected, since if cells of a strain lose the ability to display a character it is normally assumed that the progeny will do likewise. It is possible that although the egg yolk lipase is produced by cells in both positive and negative colonies, the negative colonies may contain cells unable to release the enzyme into the medium, because of some environmental condition, and, therefore, no opacity would be

visible around these colonies. Whatever the reason, the demonstration of egg yolk opacity by this method does not seem to be the ideal one for detecting lipase activity, since it is possible that a strain would produce all its colonies without the ability to demonstrate opacity in egg yolk agar, although the cells possessed the lipase enzyme. The results of the egg yolk opacity test, in which a strain, producing even a small proportion of colonies surrounded by a zone of opacity, is considered to be positive, are summarised in Table 33. In view of the results in the table, it seems likely that the lipase enzyme hydrolysing tributyrin, and that hydrolysing the fat in egg yolk to cause opacity, are quite different, but very little is known about the nature of egg yolk lipase.

Table 33

Tributyrin hydrolysis and production of egg yolk opacity by the micrococcal and staphylococcal strains

Arbitrary grouping of strains	No. of strains examined	Strains hydrolysing tributyrin	Strains producing opacity in egg yolk
Coagulase +ve staphylococci	21	21	18
Coagulase -ve staphylococci	207	202	76
Glucose oxidising micrococci	130	125	5
Micrococci unable to oxidise glucose	48	43	1

Haemolysin production

First of all, I used Haemolytic Method No.1 (page 113) to examine 260 strains for the production of haemolysins. On the assumption that complete or partial clearing of blood around colonies growing on horse blood agar was due to haemolysis, I found that many more strains were recorded as haemolytic after 72 hours at 30°C incubation (232 strains) than after 24 hours' incubation (100 strains). Many of the strains, especially those taking over 2 days to show clearing of blood, however, might not have haemolysed the red cells with an enzymic toxin, i.e. haemolysin, but could have haemolysed them by the alteration of the pH, owing to the metabolic biproducts of the bacterial cells around the growth zones. No "hot-cold" or β-haemolysin was detected in any of the plates after a further incubation period of 18 hours in the cold.

Secondly, I examined strains for their ability to produce a soluble haemolysin, apart from any pH effect, by using Haemolysin Method No.2 (page 113). Four strains of staphylococci (excluding coagulase positive strains) and 1 micrococcus strain, all of which produced haemolysis by Haemolysin Method No.1, were examined by Haemolysin Method no.2 to see (a) whether soluble haemolysins could be detected, and (b) if so, which species of red blood cells were the most sensitive to the soluble haemolysins. The results are recorded in Table 34.

Table 34

The action of micrococcal and staphylococcal haemolysins on 5 species of red cells

Strain No.*	Final dilution at which haemolytic activity can be detected in the following red cells				
	Horse	Human	Rabbit	Mouse	Sheep
78	-	-	-	-	-
133	1/8	1/4-1/8	1/16	1/4-1/8	1/16-1/32
141	-	-	-	-	-
191	-	-	-	-	-
255	0 - 1/4	-	1/8-1/16	-	1/8

* for arbitrary grouping of strains see Table 35.

From the results in Table 34, it seems as if Haemolysin Method No.2 can differentiate between haemolytic activity and a pH effect, since of the 5 strains which showed a "haemolytic" effect on horse blood agar, only 2 possessed a soluble haemolysin. While rabbit and sheep cells are the most sensitive blood cells to show soluble haemolytic activity, horse cells are also suitable for testing in Haemolytic Method No.2. Human and mouse cells were clearly not sensitive enough to detect haemolytic activity in at least one strain, and these red cells were not used for further tests. The tubes with intact red cells were kept at 4°C overnight to see if any "hot-cold" effect was apparent, but no haemolysis was noticed.

Although no experiments were done to determine the serological

nature of the soluble haemolysins, it is possible that the soluble haemolysins produced by the 2 coagulase negative staphylococci correspond to the ε-haemolysin as defined by Elek & Levy in 1950b (page 58).

A comparison was made of the amount of soluble haemolysin produced by cells of Strain no. 133 (a coagulase negative staphylococcus) when the cells were incubated under an atmosphere of 80% CO_2, and when the cells were incubated in the presence of air; no difference in the amount of haemolysin produced was detected, and therefore it seemed that, unlike the formation of α-, β- and δ-staphylococcal haemolysins, which are aided by the presence of CO_2, CO_2 was unnecessary to stimulate the formation of the soluble lysin of at least one coagulase negative strain, and hence no other strain which was tested for the presence of soluble haemolysin was cultured in an atmosphere of 80% CO_2.

Since it has been shown that horse red cells are a suitable indicator of soluble haemolysin activity, I compared the results obtained from Haemolytic Method No.1 and Haemolytic Method No.2 using horse red cells for a limited number of coagulase negative strains. If a strain produced a haemolytic titre of a soluble haemolysin greater than 1/8, the strain was considered to be haemolytic, and if the titre was less than 1/8 but greater than 1/2, the strain was considered to be weakly haemolytic, and if the titre was less than 1/2 the

strain was considered to be non-haemolytic. The results are recorded in Table 35.

Table 35

Comparison of results obtained by Haemolysin Methods Nos.1 and 2 for 18 strains of micrococci and staphylococci

Arbitrary grouping of strains	Strain No.	Haemolytic Method No.1 after incubation at 24 hours	Haemolytic Method No.1 after incubation at 72 hours	Haemolytic Method No.2 after 6 hours' incubation
Coagulase negative staphylococci	12	+	+	+
	17	-	-	+
	19	-	-	-
	50	-	+	-
	78	-	+	-
	89	+	+	-
	133	+	+	+
	148	+	+	-
	163	+	+	-
	191	+	+	-
	200	+	+	+W
	254	+	+	-
	255	+	+	+W
Glucose oxidising micrococci	65	-	+	-
	134	-	+	-
	141	+	+	-
	268	+	+	-
	270	+	+	-

+ = haemolysis +W = weak haemolysis - = no haemolysis

Four of the strains were haemolytic, and one strain non-haemolytic when examined by both methods. One strain was

haemolytic only when examined by Haemolytic Method No.2, in contrast to the 12 strains which were haemolytic only when examined by Haemolytic Method No.1. Fewer positive haemolytic strains, therefore, were recorded by Haemolytic Method No.2 than by Haemolytic Method No.1, and I maintain that Haemolytic Method No.2 is a much more accurate method of determining soluble enzymic haemolytic activity than Haemolytic Method No.1. It was found that all the 5 strains producing soluble haemolysin were glucose fermenting staphylococci; 8 other strains of staphylococci and 5 strains of micrococci failed to produce any soluble haemolysin. Some, but not all of the staphylococci, and none of the micrococci that I examined, produced a soluble haemolysin, but many more strains will have to be examined before such a conclusion can be applied to staphylococci and micrococci in general.

Haemolytic Method No.2 is an excellent method for detecting strains producing a soluble haemolysin, but since the method is time-consuming, I only examined a few strains for the character of soluble haemolysin production, and consequently I was unable to classify all 406 strains with this character.

Phage typing

The results of the phage typing are as follows:-

21 of 89 strains examined were phage typable of which
17/18 (94%) coagulase positive staphylococci were typable
0/59 (0%) coagulase negative staphylococci were typable
4/7 (57%) glucose oxidising micrococci were typable.

Of the 17 typable coagulase positive staphylococci, 5 strains belonged to Phage Group I, one strain belonged to Phage Group II, 8 strains belonged to Phage Group III, one strain belonged to Phage Group IV; 2 other strains were susceptible to phages belonging to more than one phage group, one strain sharing phages belonging to Phage Groups I and III, and the other strain sharing phages belonging to Phage Groups I, II and III.

Of the 4 typable glucose oxidising micrococci, 2 strains belonged to Phage Group III subgroup A, and 2 strains belonged to Phage Group III. From these results it seems as if some micrococci can be phage typed with *Staphylococcus aureus* phages; there does not appear to be any record of this observation in the literature.

The fact that 1 out of 18 coagulase positive strains was non-typable and that 4 out of 7 micrococci were typable indicates that phage typing is not specific for coagulase positive strains, and therefore, requires further examination before its worth in the classification of micrococci and staphylococci can be fully assessed. The character of phage typing was not used to classify all 406 strains.

ECOLOGICAL CHARACTERS

There appears to be no correlation between the sources of the strains and any of their morphological and physiological characters. For instance, not all the strains isolated from the sea, or from cheeses containing a high salt concentration, were halophilic; nor was there a higher percentage of proteolytic strains among those isolated from milk, cheese, and bacon, in comparison with strains isolated from human or animal skin. It was quite impossible to determine the source of an organism from its morphological and physiological characters alone.

It was possible, however, to show the relationship between physiological characters and source in very general terms - the most biochemically active, Gram positive, catalase positive cocci are found in, or on, the human body, where there is a high concentration of nutrients, e.g. proteins, carbohydrates, fats and vitamins, and some of these cocci cause disease in humans, e.g. *Staphylococcus aureus* : less biochemically active strains may be found on animal skin and in food, and the least active of the Gram positive, catalase positive cocci occur in habitats like dust, water and soil, where there is little nutrient.

SECTION A

MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERS OF MICROCOCCI AND STAPHYLOCOCCI

DISCUSSION OF RESULTS

DISCUSSION OF RESULTS

INTERESTING FINDINGS

After I had examined the 406 strains of micrococci and staphylococci for a total of 49 morphological and physiological characters, I naturally looked for results, which were interesting and unexpected - these are presented here -

(1) About a third of the mannitol fermenting strains are coagulase negative (page 141). Mossel (1962) and Pike (1962) found that mannitol fermentation was closely associated with coagulase production in staphylococci. Baird-Parker (1963, 1965a) did not examine his strains for mannitol fermentation but he mentioned that mannitol fermentation is one of the main characteristics of coagulase producing bacteria. My results show that there are other staphylococci, apart from the coagulase positive strains, which ferment this sugar.

(2) From the lactose and ONPG results it would appear that micrococci and staphylococci may have both lactose permease and β-galactosidase enzymes, or just β-galactosidase, or neither (page 135). McClatchy & Rosenblum (1963) found that _Staphylococcus aureus_ possessed both of these enzymes. I not only confirmed this, but showed the distribution of these enzymes in other staphylococci and also micrococci.

(3) The character of starch hydrolysis is closely associated with strains of _Micrococcus luteus_ and _M. roseus_ (page 136; Table 29). No taxonomist has put any emphasis on the character of starch hydrolysis for classification, except Pike (1962) who recorded that 76-100% of

Sarcina lutea strains and 26-100% of "miscellaneous Micrococcus spp." hydrolysed starch. Pike (1962), however, did not show that the yellow and the red pigmented micrococci unable to utilise glucose were almost the only aerobic members of the family Micrococcaceae to hydrolyse starch.

(4) The character of growth in glucose ammonium phosphate medium is closely associated with strains of yellow pigmented micrococci unable to utilise glucose i.e. Micrococcus luteus (page 137). No taxonomist has shown this association before - the difference between my results and those of other taxonomists (page 36) can probably be accounted for by my use of an inoculation system that ensured growth was dependent on the constituents of the prototroph medium, and not on the carry over of any nutrients.

(5) The character of oxidase production is closely associated with micrococci unable to utilise glucose, especially the yellow and red pigmented strains, i.e. Micrococcus luteus and M. roseus (page 122). The production of oxidase by these bacteria has not been recorded in the literature, although Steel (1962b) considered that "staphylococci" showing oxidase activity, were generally glucose oxidising strains.

(6) The character of egg yolk opacity was unstable with half of the micrococcal and staphylococcal strains, which produced opacity around some of the colonies growing on egg yolk agar (page 145). This observation does not seem to have been reported in the literature.

(7) A few micrococci were phage typed by Staphylococcus aureus

phages, and placed in phage group III (page 152). As far as I am aware there is no published work recording the phage typing of micrococci by S. aureus phages; probably the reason for this is that very few bacteriologists have tried to phage type micrococci. This result indicates that it is worth trying to phage type a wide range of micrococci in the near future.

Many of my results, however, agreed with the findings of the majority of other taxonomists - these were the observations on cell size (pages 30 and 116), colonial appearance (pages 31 and 116), appearance of cells in broth culture (pages 32 and 116), Gram's reaction (pages 32 and 116), optimum temperature (pages 33 and 117), growth at 10°C (pages 34 and 118), sodium chloride resistance (pages 35 and 119), pigmentation (pages 37 and 118), catalase production (pages 46 and 121), glucose utilisation (Tables 4 and 5 and page 133), coagulase production (Tables 2, 4 and 5 and page 141), proteolysis of milk and gelatin (pages 49 and 143) and tributyrin hydrolysis (pages 52 and 145). The results of urea hydrolysis (page 144) were found to agree with the results of other taxonomists (page 51) for the coagulase positive strains, but because there is such a difference, in published results, as to the percentage of coagulase negative staphylococci and micrococci hydrolysing urea (page 51), I considered that it was impossible to draw any significant conclusion from a comparison of my results and those of these taxonomists, especially as these taxonomists differ in their definitions of Micrococcus and Staphylococcus.

The results of haemolysin production (page 147) could not be compared with the results of other taxonomists (page 56), because of nomenclatural problems, where organisms were not sufficiently well defined, or because the species of blood haemolysed, and the type of haemolysis produced, were often not mentioned.

Since the character of growth at 40°C (page 118) has not been previously examined in strains of micrococci and staphylococci, I could not compare my results with those of other taxonomists.

Since knowledge of the taxonomy of micrococci and staphylococci has not been significantly increased by the foregoing results, which either agreed with those of other taxonomists, or could not be compared with those of other taxonomists, I have not discussed these results in detail in this section, although some of these characters have been used as main characters in previous classifications,and the significance of these main characters in classification is discussed in the next section (page 157).

THE SIGNIFICANCE OF MAIN CHARACTERS IN A CLASSIFICATION SCHEME OF MICROCOCCI AND STAPHYLOCOCCI

My next task was to determine how to use the 49 characters for classifying the strains. I could use the traditional approach to classification (page 3), and choose a few characters to be main characters for the grouping of strains, or I could use the Adansonian approach, in which all characters are treated as equal (page 3). Only if I was convinced of a character being a) unequivocally present

or absent i.e. no complicated intermediate reactions to interpret, and b) typical of the characteristics of a natural taxonomic grouping, would I consider using that character as a main character in classification. I found that only 2 characters, for which I examined the 406 strains, fulfilled these conditions. Since many taxonomists, however, have used certain characters as main characters in their classification schemes, e.g. catalase production, glucose, mannitol and lactose utilisation, acetoin production, coagulase production, phosphatase production, and pigmentation (Tables 2, 3, 4 and 5), I present in this section a detailed criticism of the use of these characters as main characters in a classification scheme of micrococci and staphylococci.

Catalase production

The genera Micrococcus and Staphylococcus are defined as being catalase positive (page 46), and all catalase negative strains are excluded from these genera, thus making catalase production a main character in the classification of Gram positive cocci. The following observations, however, indicate that catalase production may not be such an important character in the classification of the family Micrococcaceae as was previously thought:-

Three catalase negative isolates of mine shared many morphological and physiological characters with the biochemically active, catalase positive staphylococci. None of these 3 strains fits in with the definition of Aerococcus according to Cowan & Steel (1965); physiological

characters are compared in Table 36. One of these catalase negative strains (no. 6) produced a catalase isozyme band, identical to that produced by some catalase positive staphylococci (page 249), and it may well be that this strain produces small amounts of catalase undetectable by the physiological catalase test (page 101). It is also interesting to note that there are, in the literature, reports of catalase negative staphylococci (Lucas & Seeley, 1955; Jensen, 1963; Solomon & San Clemente, 1963).

These 3 strains, therefore, appear to be in a group separate from staphylococci, micrococci and aerococci, and the taxonomic position of the group is somewhere between the staphylococci and the aerococci, but I would not exclude them from the Micrococcaceae because of the apparent loss of one enzyme.

Table 36

Comparison of characters exhibited by Aerococcus viridans and the 3 catalase negative strains

Character	Aerococcus viridans	Catalase negative strains		
		strain no. 6	strain no. 31	strain no. 89
Catalase production	-	-	-	-
Glucose fermentation	+	+	+	+
Aerobic utilisation of mannitol	variable	+	+	+
Mannitol fermentation	unknown	+	+	+
Acetoin production	-	+	+	+
Phosphatase production	-	+	+	-
Gelatin liquefaction	-	-	-	-
Growth in 6.5% NaCl	+	+	-	-
Type of haemolysis in horse blood agar	α	-	-	β
Habitat	air, dust	human skin	human urine	pus from infected finger
+ = positive reaction				
- = negative reaction				

The utilisation of carbohydrates

Since the characters of glucose, mannitol, and lactose utilisation, starch hydrolysis, growth in glucose ammonium phosphate medium and acetoin production are dependent on the carbohydrate metabolism of cells of micrococci and staphylococci, they are dealt with in this

section. Even although the characters of starch hydrolysis and growth in glucose ammonium phosphate medium have not been used as main characters in classification schemes in recent years, they are included in this section because organisms producing these characters are of importance in discussing the use of glucose utilisation as a main character in the classification of micrococci and staphylococci.

<u>Glucose and mannitol utilisation</u>: The characters of fermentation and oxidation of glucose and mannitol have been used by taxonomists as important criteria in classification schemes of micrococci and staphylococci (page 39), and, since 1955, glucose fermentation has been regarded by all taxonomists as the sole criterion for the separation of the genus *Micrococcus* from the genus *Staphylococcus* (page 14). For these reasons it is important that the utilisation of glucose, and other "sugars", under both aerobic and anaerobic conditions, by strains of micrococci and staphylococci can be determined unequivocally.

Unfortunately I discovered that there were several problems involved in testing strains for these characters.

First of all, there was the technical problem of obtaining anaerobic conditions by the use of liquid paraffin overlay; I found that it was impossible to ascertain whether or not fermentation had occurred in every strain by this method (page 124). Although many taxonomists used liquid paraffin or vaspar overlay to create anaerobic conditions in the determination of fermentation of "sugars" (Table 10), I found that

incubation of cultures in an atmosphere of hydrogen in an anaerobic jar was a much more effective method of achieving anaerobic conditions (page 124).

Secondly, I found that different media e.g. Glucose Agar Media Nos. 1 and 2, Glucose Broth Media Nos. 1 and 2, gave different results when the action of the same strains was examined in them (page 124); I was able to classify as a Micrococcus a strain recorded by Baird-Parker (personal communication) as a Staphylococcus, and also able to classify as Staphylococcus 2 strains recorded by Baird-Parker (personal communication) as Micrococcus, on the basis of glucose fermentation when I used a different glucose agar and a different method of achieving anaerobic conditions from Baird-Parker (1963).

Thus it is by no means easy to determine whether a strain oxidises or ferments a "sugar", and standardised conditions must be used by all taxonomists if fermentation of glucose is to be recognised as the criterion for separating Staphylococcus from Micrococcus.

It is not only the problem of standardising conditions that raises difficulties with these tests, however, but also the interpretation of colour changes taking place in the test tubes when strains are examined for fermentation or oxidation of "sugars" in a complex "sugar" medium. A complex medium must be used for the detection of "sugar" fermenting and oxidising strains of micrococci and staphylococci, since, as Jones et al., (1963) point out, some strains cannot grow in a simple medium without the requirement of certain growth factors such as biotin and uracil.

Taxonomists, as Evans (1955) and Baird-Parker (1963, 1965a), have regarded a visible change in colour of the pH indicator in an inoculated "sugar" medium as the sole means of recognising fermentation and oxidation, when cultures have been incubated under anaerobic conditions and aerobic conditions respectively; the colour change in the pH indicator is presumed to be due to acid arising from carbohydrate metabolism.

Yet anomalous reactions were observed when a few micrococcal strains growing in Glucose Agar Medium No. 1 under aerobic conditions, produced transient changes in colour of the pH indicator. These strains produced in the medium yellow zones indicating weak acid production, and after further incubation the cultures became purple in colour once more, thus indicating an increase in pH, to give apparently negative reactions (page 126). Shaw et al. (1951), Evans et al. (1955), and Baird-Parker (1963) were also aware of such transient changes of pH during growth of strains in complex "sugar" media.

The only explanation I can give for these anomalous reactions is that small amounts of acid are produced by the strains, and at a later stage of growth the acid is neutralised by alkaline products of metabolism. This observation would indicate that many metabolic reactions occur during growth in a complex "sugar" medium, and these would render the oxidation - fermentation test, as it is at present, unsuitable for detecting small amounts of acid produced from "sugars". The manometric experiments of Nunheimer & Fabian (1942) and Rosypal

& Kocur (1963) seem to bear this last point out. These authors found that the apparently biochemically weak strains of Micrococcus luteus and other Micrococcus species could not only oxidise glucose but many other sugars as well (page 42). Oxidation was measured by O_2 uptake, is a more direct method of detecting metabolism of sugars than the change in colour of a pH indicator by the acidic end products of metabolism.

Lactose utilisation: Glucose is generally considered to be the simplest sugar for cells to metabolise. Yet 4 of 287 strains, capable of producing acid from lactose under aerobic conditions within 2 days, apparently could not produce acid from glucose in Glucose Agar Medium No. 2 under the same conditions (page 135). If these 4 strains cannot hydrolyse glucose to give acid, it is difficult to understand how they can hydrolyse lactose, a disaccharide consisting of glucose and galactose units. Baird-Parker (1965a) found that 12% of Micrococcus luteus strains utilised lactose aerobically, but he did not comment on the fact that these strains were classified by him as micrococci unable to produce acid from glucose.

Propably the most rational explanation of these apparently contradictory results is that the 4 strains producing acid from lactose also produce small amounts of acid from glucose, but small amounts of acid are not easily detected in Glucose Agar Medium No. 2, because either the constituents of the medium or alkaline products of metabolism neutralise the acid.

Starch hydrolysis: Starch is a polymer of glucose, but most of the strains

hydrolysing starch are the micrococci apparently unable to utilise glucose, in particular strains of Micrococcus luteus and M. roseus (page 136). Starch hydrolysis by strains of Micrococcus luteus (Sarcina lutea in the nomenclature of Pike, 1962) has also been recorded by Kocur & Martinec (1962) and Pike (1962), and in addition starch hydrolysis by strains of Micrococcus roseus has been recorded by Shaw et al. (1951) and Kocur & Martinec (1962), but none of these authors commented on the significance of strains breaking down starch but not, apparently, glucose. If any of the end products of starch hydrolysis are utilised by these strains, they must also utilise glucose, since starch is built up with glucose molecules. It seems strange that some strains would break down starch without using any of the end products, and therefore, these reactions appear to me to be anomalous.

Probably the most satisfactory explanation for these anomalous reactions is that all the starch utilising strains are able to utilise glucose, but as stated before (page 163), the oxidation-fermentation test in a complex glucose medium is not sensitive enough to detect small amounts of acid production from the glucose.

Growth in glucose ammonium phosphate medium: Strains capable of growing in glucose ammonium phosphate medium must be able to utilise both the ammonium salt (the sole source of nitrogen), and also the glucose (the sole source of carbon). It was noted however, that only 9 of the 16 strongly prototrophic strains were able to produce any colour changes in the medium, indicating acid production (page 137); the other 7 strains

must have utilised the sole carbon source, glucose, but they either failed to produce sufficient acid or the acid produced was neutralised, so that no colour change in the indicator was visible. Both Hill (1959) and Baird-Parker (1963) noticed that some strains of micrococci and staphylococci which grew in glucose ammonium phosphate medium apparently did not produce any acid; neither author, however made any deductions from these observations.

It was also noted that 15 of the 16 strains capable of growing in glucose ammonium phosphate medium were strains of yellow pigmented micrococci unable to utilise glucose i.e. Micrococcus luteus (page 137), and therefore these strains should be able to utilise glucose. Yet these strains were apparently unable to produce acid from glucose in Glucose Agar Medium No. 2. although the same pH indicator was present in both media. Baird-Parker (1965a) records that some strains unable to produce acid from glucose in Glucose Agar Medium No. 1 grew in glucose ammonium phosphate agar, but he makes no comment on these apparently anomalous results.

From this comparison of results it would seem that strains which can grow in a simple medium, like glucose ammonium phosphate agar, produce acid more easily than when they are growing in a complex medium, like Glucose Agar Medium No. 2. Either some of the components of the complex medium (e.g. tryptone and yeast extract) buffer small amounts of acid produced, or the complex medium allows a greater number of metabolic pathways to be produced by the strains, and consequently there

is a greater likelihood that alkaline compounds would be excreted into the medium, and neutralise any acid formed.

Acetoin production: The first problem that I found was that acetoin production by the 406 strains was different in the 2 media used, Acetoin Medium No. 1 (glucose phosphate medium) and Acetoin Medium No. 2 (unbuffered glucose broth) - the phosphate appears to influence the acetoin production, but the mechanism of the phosphate on the acetoin production pathway is unknown. The taxonomist has to decide which of the 2 media is best for detecting acetoin producing strains of micrococci and staphylococci - a difficult task.

Secondly, I noticed that some of the strains produced anomalous biochemical reactions difficult to interpret, i.e. acetoin was produced by some strains which only oxidised glucose or did not utilise glucose at all (including 2 strains of *Micrococcus luteus* and 1 strain of *M. roseus*). Baird-Parker (1965a) found that acetoin was produced by 47% of glucose oxidising micrococci, and he recorded the fact that 10% of his strains of *Micrococcus luteus* and *M. roseus* (micrococci apparently unable to utilise glucose) were weakly acetoin positive. Baird-Parker (1963, 1965a) used acetoin production as a main character in his classification scheme, but he assumed that the acetoin producing pathway was the same for strains fermenting glucose, strains oxidising glucose and strains unable to utilise glucose at all. I comment on this assumption since the known pathways of acetoin production require pyruvate as a starting point, and pyruvate is normally produced as a result of glucose fermentation; strains

not known to ferment glucose must obtain pyruvate by different means, such as deamination of alanine, or acetoin may be formed directly by the dehydrogenation of butylene glycol. Not only is it difficult to relate acetoin production by strains to their glucose reactions, but it appears that the acetoin producing system, at least in Staphylococcus aureus, is only developed if acid is produced by strains during growth (Watt & Werkman, 1951). Clearly strains incapable of producing acid from glucose in a glucose medium could not produce acetoin, and yet I and Baird-Parker (1963) have recorded from our respective physiological tests, that a few strains of micrococci are capable of producing acetoin without producing acid from glucose, even under aerobic conditions.

These anomalous reaction can best be explained by the assumption that the acetoin producing strains produce at least a little acid from the glucose in the acetoin media, but small amounts of acid produced by the same strains in a complex glucose medium are rapidly neutralised before the colour of the pH indicator can be changed.

The use of glucose, mannitol and lactose utilisation, and acetoin production as main characters - conclusions: From these observations I consider that all Micrococcus strains utilise glucose, and probably other "sugars," when they are grown in the appropriate "sugar" media, but in the course of metabolism little acid is produced, and this is neutralised, more in a complex medium than in a simple medium, by the alkaline products, arising from other metabolic pathways during the growth of the strains.

It seems to me that the detection of acid from glucose and other sugars under aerobic and anaerobic conditions should not be used as major characters in the classification of Gram positive, catalase positive cocci. I have shown that the complex medium Glucose Agar Medium No. 2, proposed by the Subcommittee on Taxonomy of Staphylococci and Micrococci (1965b) to be the standard medium used for the separation of strains into the genera *Staphylococcus* and *Micrococcus*, is unsatisfactory, since some strains growing in the medium produce acid not detectable by a colour change in the pH indicator. Kocur & Mortensen (1967) and Gibson (1967) have also found that acid production by strains of Gram positive, catalase positive cocci, growing in this medium, is unsatisfactory as a criterion for dividing these strains into 2 clear-cut groups, *Micrococcus* and *Staphylococcus*.

Oxidation and fermentation of carbohydrates is probably best detected by examining washed respiring cells of the strains to be classified in a Warburg apparatus, but the method is time-consuming and it is unlikely that it would be used for standard classification purposes. An alternative method of testing for the utilisation of a carbohydrate is to test for the disappearance of that carbohydrate from a medium during growth of an organism, but no such test is yet in existence.

Thus, at the moment, there is no simple, satisfactory way for determining whether a Gram positive, catalase positive strain is fermentative, oxidative or unable to utilise "sugars" at all, and

therefore fermentation of glucose should not be the sole test for classifying these strains into the genus Micrococcus or the genus Staphylococcus; acid production of carbohydrates should be given equal weight with other characters in any classification scheme of these organisms.

Similarly, because of the lack of a suitable acetoin medium, and the lack of knowledge of the acetoin producing pathway in micrococci and staphylococci, I consider that it would be unwise to use acetoin production as a main character for classifying these organisms.

Coagulase production

Since a few coagulase negative members of the Micrococcaceae have been shown to cause disease, the character of coagulase production can no longer be considered to be uniquely associated with pathogenic staphylococci (page 54), and also the character cannot, by itself, be considered to be the sole criterion of the species Staphylococcus aureus (Baird-Parker, 1965a; Kocur et al., 1966). Baird-Parker (1965a) is of the opinion that S. aureus is defined as being positive in 2 of the following 3 biochemical reactions - coagulase and phosphatase production, and mannitol fermentation. Kocur et al. (1966) found that 5% of coagulase negative strains could produce one or more of the α-, β-, or δ- haemolysin, all formerly thought to be uniquely associated with coagulase positive Staphylococcus aureus strains.

I found, in my characterisation of the 406 strains, that a few coagulase negative strains possessed physiological characters typical

of the coagulase positive staphylococci e.g. acetoin and phosphatase production and mannitol fermentation; in addition a few coagulase positive staphylococci lacked the characters of mannitol oxidation and fermentation. I consider, therefore, that it is unwise to treat coagulase production as the sole character for defining a taxonomic group i.e. Staphylococcus aureus.

Phosphatase production

Several taxonomists have used the production of phosphatase as a character in classification schemes of micrococci and staphylococci; it is clear that the incubation time is an important factor in determining the production of phosphatase by these organisms (page 55). Since I used the same incubation time as Baird-Parker (1963, 1965a), I can compare my results with his (Table 37).

Table 37

The comparison of Baird-Parker's (1963, 1965a) results, and my results of the percentage of micrococcal and staphylococcal strains producing phosphatase

Authors		Arbitrary grouping of strains			
		Coagulase + ve staphs.	Coagulase - ve staphs.	Glucose oxidising micrococci	Micrococci unable to use glucose
Baird-Parker (1963)	No. of strains examined	24	546	687	13
	% age phosphatase + ve	100%	78%	1%	30%
Baird-Parker (1965a)	No. of strains examined	80	81	303	48
	% age phosphatase + ve	100%	40%	37%	22%
Results presented in this thesis (page 142)	No. of strains examined	21	207	130	47
	% age phosphatase + ve	100%	72%	76%	30%

There is agreement between my results and Baird-Parker's (1963, 1965a) results as far as the percentage of phosphatase positive strains in 2 of the 4 arbitrary groups - the coagulase positive staphylococci and the micrococci unable to utilise glucose - is concerned. It is quite clear, however, from the results presented in Table 37 that most of the coagulase negative staphylococci and most of the glucose oxidising micrococci that Baird-Parker (1963) studied were different in phosphatase producing ability from the strains from the same 2 arbitrary groups that Baird-Parker (1965a) and I studied. Baird-Parker (1965a) has not commented on the difference in the number of phosphatase producing strains in these 2 groups in his 2 classifications of staphylococci and micrococci (1963 and 1965a). Even although the strains examined in the 3 schemes presented in Table 37 were different they were all obtained from broad spectra of sources, and I would expect a closer correlation of findings in the coagulase negative staphylococci and the glucose oxidising micrococci than is the case, if phosphatase producing ability is thought to be characteristic of subgroups within these 2 groups. In fact the character of phosphatase production cannot be correlated with any other morphological or physiological character either from my results or from Baird-Parker's (1963; 1965a), and Baird-Parker's (1963; 1965a) decision to divide strains of micrococci and staphylococci into subgroups on the basis of phosphatase producing ability, among other characters, is an arbitrary one.

Therefore, I can find no reason from published results or my own results for any taxonomist to regard phosphatase producing ability as a main character in a classification scheme of micrococci and staphylococci.

Pigmentation

The use of pigment production as a character in published classification schemes of micrococci and staphylococci is reviewed on page 37 ; pigment production is dependent on the composition of the media used and the intensity of the light, and, in addition, pigmented strains can sometimes lose the ability to produce the pigment. I found that pigmentation did not correlate well with any other physiological character in the 406 strains that I examined, except in the group of biochemically weak micrococci (page 119 ; Scheme 1, pocket inside front cover).

In particular, it is worth commenting on the taxonomic significance of pigmentation in the following 2 organisms. *Micrococcus violagabriellae* has a unique violet pigment, yet the strain that I studied possessed all the other physiological characters typical of strains of *Staphylococcus epidermidis*, although it is unusual in being pathogenic for humans (Castellani, 1955). I, therefore, confirm the opinions of Kocur & Martinec (1963a) and Baird-Parker (1965a) that *M. violagabriellae* is, in fact, a pathogenic, violet pigmented strain of *Staphylococcus epidermidis*. Black pigmented *S. flavocyaneus* possessed many physiological characters in common

with *Micrococcus luteus*, and, therefore, I agree with Kocur & Martinec (1963a) that *S. flavocyaneus* should be reclassified as a strain of *M. luteus*. The production of unusual pigments by strains of micrococci and staphylococci, therefore, does not necessarily indicate that these strains possess other unusual physiological characters, and pigmentation, for these strains, should not be used as a main character in their classification.

Most strains (36/48) in the group unable to utilise glucose were either yellow or red pigmented micrococci (page 119). Unfortunately it is difficult to compare the production of red and yellow pigments with other physiological characters, because of the relative biochemical inactivity of strains in this group. The production of yellow and red pigments, however, correlates well with electrophoretic characters (Scheme 2; pocket inside back cover).

In classification of micrococci and staphylococci, therefore, the use of pigmentation as a main character is not justified except in the division of the group of micrococci unable to utilise glucose.

From the results of my examination of the 49 morphological and physiological characters of the 406 strains, I found that yellow and red pigmentation could be used to divide the micrococci unable to utilise glucose into 2 natural groupings - *Micrococcus luteus* and *M. roseus* respectively - these are the only characters able to satisfy the conditions laid down on page 157 for their use as main characters in a classification scheme.

Therefore, since so few characters could be used validly as main characters in an arbitrary classification scheme of the 406 strains of micrococci and staphylococci, I had to classify my strains by the Adansonian way.

SECTION A

THE ADANSONIAN CLASSIFICATION SCHEME OF MICROCOCCI AND STAPHYLOCOCCI BASED ON MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERS - SCHEME 1

THE ADANSONIAN CLASSIFICATION SCHEME OF THE 406 STRAINS BASED ON MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERS

Introduction

Originally I intended to take all the morphological and physiological characters of the 406 strains, and analyse these, with the use of a computer, in order to find the similarity values of the strains, and from this information, I would construct a numerical taxonomic Adansonian classification scheme. Unfortunately, the time taken to prepare the material for the computer was greater than I had originally anticipated - the greatest number of strains to be compared by computer analysis in one run is, to my knowledge, less than 400. Therefore, although I am hoping to create a numerical taxonomic classification scheme, based on morphological and physiological characters in the near future, I am unable to present this in the thesis. Instead, I constructed an Adansonian classification scheme without the use of a computer, in which a selected number of the morphological and physiological characters were treated as equal for taxonomic purposes.

Classification results

All the 406 strains were compared for their 49 morphological and physiological characters, and I found that the strains varied considerably in the number of characters they possessed i.e. there are many intermediate groups of strains between the coagulase producing, glucose and mannitol fermenting staphylococci, and the yellow and red pigmented, biochemically weak micrococci.

Very few of the 406 strains shared identical combinations of characters, and if the complete characterisation of each individual strain was given in a table, the tabulated information would extend for pages; this, I feel, would be of limited value, since there would be approximately 400 different combinations of characters, each one of which could be called a "natural grouping". The fact that most strains of micrococci and staphylococci fall into very many groupings can, I think, be equally well shown in a scheme of smaller proportions. The 406 strains were classified, as shown in Scheme 1 (pocket inside front cover), by the Adansonian principle on the basis of the results of 14 physiological characters, which have been used as main characters for the classification of micrococci and staphylococci by other taxonomists (Tables 2, 3, 4 and 5) - catalase production, white, yellow, golden, red, and other pigment production, glucose oxidation and fermentation, mannitol oxidation and fermentation, phosphatase, acetoin, and coagulase production, and starch hydrolysis. The character of yellow pigmentation includes all shades of yellow excluding golden. Each of these characters is treated as being equal for classifying strains. Every different combination of characters constitutes a different group in Scheme 1, although I have used pigmentation as a grouping character only for the micrococci unable to utilise glucose, and for micrococci in Baird-Parker's (1965a) Micrococcus subgroup 6, since it is only for these organisms that pigmentation has been shown to be an important

criterion for classification in recent years (Kocur & Martinec, 1962, Baird-Parker, 1963, 1965a). The symbols relating to the characteristics of a strain, or a group of strains, is given in Scheme 1; a negative character is recorded as a blank zone.

All together, there are 52 physiological groups in Scheme 1. On the characterisation of the 406 strains by the 14 physiological characters there are only 2 groups of strains which appear to stand out:- 1) the catalase negative strains, and 2) the coagulase positive strains, and even these groups are not clear cut. Most of the 406 strains are, in fact, in a large heterogeneous group, and there is no division of these strains into 2 well defined main groups, which would correspond to the genera Micrococcus and Staphylococcus. Even if a division of the strains into 2 physiological groups was desirable, I would not choose glucose fermentation as the dividing character (c.f. Evans et al., 1955; Baird-Parker, 1965a), but glucose oxidation, since the latter character seems to separate the group of biochemically active strains from the group of biochemically weak strains (Scheme 1). Nevertheless, there are many intermediate groupings between the biochemically active, and the biochemically weak groupings. Therefore, I am of the opinion that the micrococci merge into the staphylococci, and it would appear that no one morphological or physiological character can separate them.

Since it is now generally recognised that Micrococcus is a valid generic name (page 14), I consider that all Gram positive, catalase

positive cocci should be placed in the genus *Micrococcus* i.e. all the strains that I classified, excluding the 3 catalase negative ones, (403) should be members of the genus *Micrococcus*. In this statement I agree with the taxonomic position taken by Breed (1954) and Hucker (1954) in the 6th edition of Bergey's Manual (1948) and Van Eseltine (1955).

Another important conclusion arising from this classification is that no recent classification scheme, based on morphological and physiological characters can be used for classifying all the 403 strains of Gram positive, catalase positive cocci into species, groups, or subgroups; even the most comprehensive of these schemes, that of Baird-Parker (1963, 1965a), could be used to classify only 307 of the 403 strains (Scheme 1). Baird-Parker's (1963, 1965a) 6 *Staphylococcus* subgroups and 7 *Micrococcus* subgroups are shown in Scheme 1 opposite the combination of characters that defines his subgroups. Only a simple scheme, like that of Shaw *et al.*, 1951 (Table 3), could be used to classify all the strains but this classification scheme is no longer recognised by taxonomists. Clearly an unsatisfactory state exists in the taxonomy of micrococci and staphylococci, if about one quarter of these strains cannot be classified by any recognised classification scheme based on morphological and physiological characters.

Since I did not achieve a satisfactory classification of the 406 micrococcal and staphylococcal strains into natural groupings with morphological and physiological characters, I decided that, owing to

the success of taxonomists in classifying various types of bacteria by the use of electrophoresis (Table 38), I would examine the 406 strains for electrophoretic characters (page 181).

SECTION B

REVIEW OF THE LITERATURE

ELECTROPHORESIS AS A TAXONOMIC TOOL

The technique of electrophoresis

Tiselius (1937a) was the first person to use the technique of electrophoresis to separate a mixture of proteins; using moving boundary electrophoresis in free solution, he showed that serum contained several protein components. Although accurate mobilities of proteins can be measured with the moving boundary method, it has the disadvantages of requiring a large size of sample (50 mg.), poor resolution of protein components, and a large and complex apparatus.

The moving boundary method has been since superseded by zone electrophoresis in solid supporting media for the separation of soluble fractions of plant and animal tissues into protein components, which are stained in the media by a specific protein stain. Zone electrophoresis has advantages over the older method, as it gives rapid separation of components in small amounts of sample material in a relatively simple apparatus. Filter paper was the original material used for the supporting medium (Cremer & Tiselius, 1950), but agar gels (Gordon et al., 1950) and cellulose acetate membranes (Kohn, 1957) are now widely used. These inert supporting media are used, since they minimise diffusion during separation, and interact with proteins as little as possible, but they do not give complete resolution of proteins.

Zone electrophoresis in strong gels i.e. gels exerting a sieving

action upon the molecular size of proteins, in addition to the separation dependent on electric charge, resolves plant and animal tissue extracts into protein components much better than electrophoresis in inert media. The first of these gels were composed of starch (Smithies, 1955), but more recently another medium - that of polyacrylamide - for gel electrophoresis was introduced by Raymond & Weintraub (1959). Polyacrylamide gels have certain advantages over starch gels for zone electrophoresis - polyacrylamide gels can be made more quickly, can be used over a wider concentration range, can be used with a wider range of buffers, and are less fragile to handle; in addition, polyacrylamide gels are completely transparent, unlike starch gels, which are opaque.

Enzymes were first demonstrated in starch gels, by Hunter & Markert (1957), who electrophoresed human tissue extracts, and stained the gels for zones, usually termed bands, of esterase activity. Although esterases can hydrolyse a number of organic esters, esterase activity is normally defined as the ability of an enzyme to release naphthol from a substrate composed of an organic acid ester of α-naphthol e.g. α-naphthol acetate. Many other enzymes such as lactic dehydrogenases (Markert & Moller, 1959), aminopeptidases (Lawrence <u>et al</u>., 1960) peroxidases (Hughes, 1961), and catalases (Paul & Fottrell, 1961), have been detected in starch gels after electrophoresis of human and animal tissue extracts.

Multimolecular forms of enzymes, termed isozymes by Markert & Moller

(1959), have been recorded in zone electrophoretic studies of bacterial extracts, and extracts of plant and animal tissues. The mobility of enzymes, electrophoresed across starch or polyacrylamide gels, depends on their molecular size, shape, and also their charge; isozymes occur where enzymes with the same function are different in one or more of these 3 physical characters.

<u>The use of zone electrophoresis in classification of bacteria</u>

In taxonomic studies of bacteria, starch and polyacrylamide gels are the supporting media that have largely been used for zone electrophoresis. Separation of bacterial proteins by electrophoresis was first noticed by Rosenkast & Clausen (1962), and in the same year Norris detected esterase isozyme bands in electrophoretically-run disintegrates of 5 <u>Bacillus</u> species. Since then many taxonomists have tried to classify bacteria by zone electrophoresis and summaries of their results are listed in Table 38 . All these authors seem to find a correlation between the grouping of strains by their electrophoretic protein or enzymic patterns and grouping by other criteria i.e. morphological, physiological or serological characters .

<u>Criticism of the applications of zone electrophoresis for the classification of strains in the family Micrococcaceae</u>

Little work has been done to try to classify strains within the family <u>Micrococcaceae</u> by the technique of zone electrophoresis. The study of <u>Staphylococcus aureus</u> by Rosenkast & Clausen (1962) was carried out by electrophorising 3 autolysed strains (each of which was a

Table 38

Summary of classification schemes of bacteria by zone electrophoresis

Authors	Group of organisms studied	Gel support medium	Cell constituents examined	Results
Rosenkast & Clausen(1962)	*Staphylococcus aureus*(3)*	agar	protein	Division of 3 strains of *S.aureus* into 3 groups which correlate with Cowan's (1939) serological groups.
Norris (1962)	5 *Bacillus* sp. (5)	starch	esterases	5 main esterase groups; small celled species contained a band absent in large celled species.
Fowler *et al.* (1963)	pleuro-pneumonia - like group (12)	starch	protein	12 protein patterns which have a certain correlation with serological groups; electrophoretic patterns could be used to subdivide serological groups.
Norris (1964)	*Bacillus thuringiensis* (46)	starch	esterases	12 esterase patterns; close correlation between serological classification and that based on esterase analysis.
Cann & Willox (1965)	4 *Mycobacterium* sp. (49)	starch	esterases catalases	4 main types of esterase patterns which correspond to the biochemical division of the genus Mycobacterium into the 4 species.

* nos. in brackets = strains

Table 38 (continued)

Authors	Group of organisms studied	Gel support medium	Cell constituents examined	Results
Lund (1965)	*Streptococcus* group D (23)*	polyacrylamide	protein esterases	4 main protein & esterase groups which correspond to 3 species which compose the group D streptococci.
Baird-Parker (1965a)	staphylococci and micrococci (46)	starch	protein	some protein patterns characteristic of Baird-Parker's (1963, 1965a) groupings.
Robinson (1965a)	20 *Corynebacterium* sp. (24)	starch, polyacrylamide	esterases catalases peroxidases protein	15 patterns which correlate with habitat viz. human, animal, and plant pathogens. *C. pyogenes* rejected from genus.
Robinson (1966c)	3 *Microbacterium* sp. (25)	starch	esterases catalases	11 esterase-catalase patterns; close correlation between these patterns and the 3 species.

* nos. in brackets = strains

representative of one of Cowan's, 1939, 3 serological groups) on agar, and then staining for protein. Although these authors saw that each strain had one distinct protein band with a different mobility from the other bands, Rosenkast & Clausen (1962) could hardly be justified in concluding that Staphylococcus aureus could be divided into 3 protein groups, which correspond to Cowan's (1939) serological groups. The reasons for my criticism of these conclusions are as follows:-

1) they used only 3 strains, which is a very small number for any taxonomic study.

2) the cells of the strains were allowed to autolyse before electrophoresis was carried out; many changes could take place in the cells that would alter the nature and the numbers of proteins during this process.

3) the authors detected only 1 distinct protein band for each strain. The more recent work of Lund (1965), Baird-Parker (1965a), and Robinson (1966a) demonstrated, however, that all their respective strains of bacteria (Table 38) had complex electrophoretic protein patterns. That these last authors found many protein bands in every strain is probably because they used starch or polyacrylamide gels as supporting media, whereas Rosenkast & Clausen (1962) used an agar gel, which is not the best supporting medium for zone electrophoresis. It is possible that after autolysis of the cells, the 3 proteins which remained, and were detected by Rosenkast & Clausen (1962), were

Cowan's (1939) 3 agglutinogens, but more strains must be examined and better supporting media for zone electrophoresis used before this statement could be made with confidence.

Baird-Parker (1965a) examined 46 strains of micrococci and staphylococci for electrophoretic protein patterns, but he does not show any photographs or diagrams in his paper. He found that patterns were "characteristic" of *Staphylococcus* subgroups II, III, IV and V and *Micrococcus* subgroup 8 (Table 5), but *Staphylococcus* subgroups I and VI apparently gave a variety of patterns within a subgroup. It seems to me that Baird-Parker (1965a) is uncertain of the significance of his results and his comments are not very helpful towards the solution of the taxonomic problems of micrococci and staphylococci.

Therefore, at present, there is no satisfactory electrophoretic classification scheme of micrococci and staphylococci.

SECTION B

ELECTROPHORETIC ANALYSIS OF CELL CONTENTS OF STRAINS OF MICROCOCCI AND STAPHYLOCOCCI

MATERIALS AND METHODS

PREPARATION OF CELL CONTENTS FOR ELECTROPHORESIS

Cells of all 406 strains of micrococci and staphylococci were grown in batch cultures, harvested, and then disintegrated to release the soluble contents of the cells. In preliminary experiments it had been shown that the soluble cell contents carry practically all the enzymic activity, which can be detected in gels, at least as far as esterases and catalases are concerned, and the slight activity occurring in the disintegrated cell wall fragments is most likely to be due to the contamination of the cell wall fragments with the cell contents.

Batch culture of cells

Method 1. - Large scale broth culture

To harvest large quantities of cells for electrophoretic analysis of the cell contents, I chose to adopt a large scale broth culture method at the beginning of my work. This was because the Braun M.S.K. homogeniser (Shandon Scientific Co.Ltd., London) for disintegrating bacterial cells, was not then available, and the volume of cells required for electrophoretic experiments was therefore not known. I obtained packed cells of 110 different strains of micrococci and staphylococci by this method.

a) The medium

Several media of different chemical composition were examined for their suitability for growing strains of micrococci and staphylococci in large scale broth culture. The following medium was the medium of choice:-

% $\frac{w}{v}$ in water	Nutrient broth (Oxoid)	0.8
	Yeast extract (Oxoid)	0.5
	NaCl	0.5
	K_2HPO_4	0.5
	Glucose	0.2

The constituents were dissolved in hot tap water, and 4 litres of the medium were placed into 5 litre conical flasks, and the pH adjusted to 7.

b) Sterilisation of medium

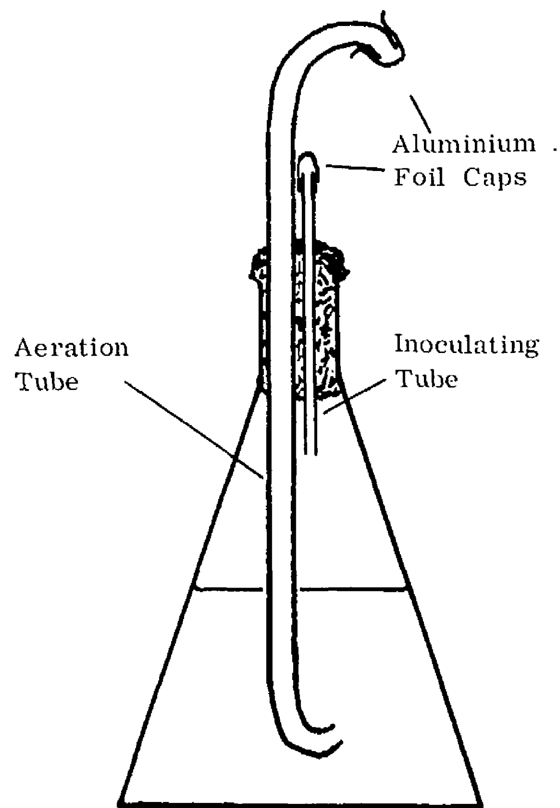
The flasks were prepared as in Diagram I with tubes for inoculation and aeration. The ends of these tubes, which were exposed to the atmosphere, were covered with aluminium foil. As rubber bungs perish on autoclaving, cotton wool plugs were used to seal the necks of the flasks. The whole upper parts of the flasks, including the projecting parts of the tubes, were covered with kraft paper to prevent the cotton wool becoming soaked by steam during the autoclaving process. The 4 litres of medium in the flasks were first of all heated for 60 minutes at 100°C in a Koch, and then autoclaved for 60 minutes at 10 lb./sq. in. of steam. This long autoclaving time, which was required to ensure complete sterilisation of these large volumes of medium, did not appear to affect the 0.2% glucose, but a little precipitation of the phosphate and yeast extract occurred. After autoclaving, the flasks were cooled to 37°C and kept at that temperature for two days to ensure that no contamination was present.

c) Inoculation

A tube of glucose phosphate medium (Mackie & McCartney, 1960; page 218) was inoculated with a pure culture of a strain and incubated at 37°C

Diagram I

Flask for large scale broth culture



5 litre flask
containing 4 litres of
medium

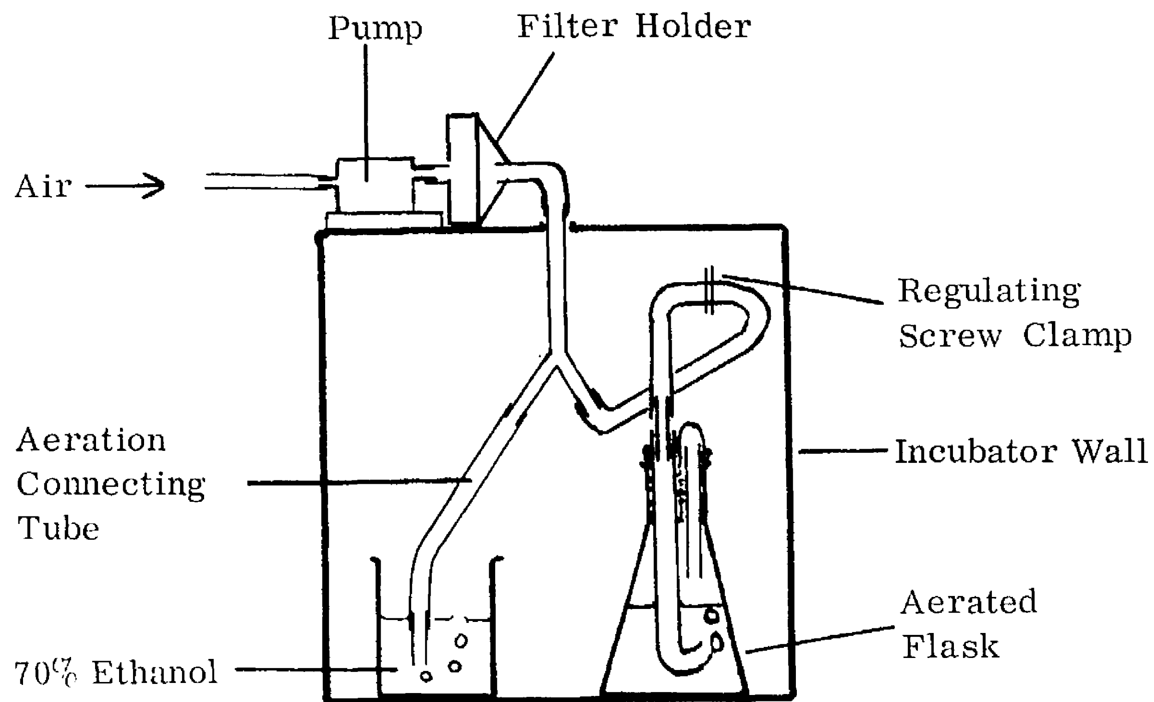
for 18 hours. After this time the growth was microscopically checked for contamination by Gram stain; if the preparation showed no contamination, about 2 ml. was aseptically transferred by pasteur pipette into the medium in a 5 litre flask through the inoculating tube (Diagram I), the aluminium cap being replaced on the tube after inoculation.

d) Aeration

A simple but effective pump, which could be run for weeks without overheating, (Hyflo Model C, Medcalf Bros. Ltd, available from Griffin & George, England) was used to aerate the cultrues. A filter holder, into which a cellulose acetate bacteriological airflow filter could be inserted, was attached to the outflow of the pump. The air flow from the filter was divided into two by a glass "Y" junction so that two flasks could be aerated and incubated at the same time. The filter holder, glass "Y" junction and plastic aeration connecting tubes were autoclaved for 15 minutes at 15 lb./sq.in. of steam, and then a sterile cellulose acetate filter was aseptically placed into the filter holder, which was connected to the pump. Both the pump and the filter holder were placed on top of an incubator in such a way that a tube containing sterile air could pass through the ventilation hole of the incubator and into the incubating flasks. Screw clamps were placed on the plastic tubing connecting the airflow to the flasks in order to regulate the flow of air entering each flask (Diagram II). The aeration tube dipping into the media gave sufficient dispersion of air to aerate the cultures with a minimum of frothing, and the inoculation tube (Diagram I) served as a gas escape for excess air and also for the carbon dioxide

Diagram II

Incubating conditions for 5 litre flasks



Incubator at 35°C

evolved during bacterial respiration. The transfer of the aeration connecting tubes from one flask to another presented a contamination problem. This, however, was overcome by keeping the pump in continuous operation so that a positive air pressure was maintained within the aeration connecting tubes, and the end of an aeration connecting tube was placed in a beaker of ethanol 70% in water - this concentration is known to be bactericidal (Epstein, 1897) - and air allowed to bubble through the tube for 10 minutes (Diagram II). When the next flask for incubation was ready the aeration connecting tube was quickly and aseptically removed from the alcohol and fitted to the flask so that aeration could begin at once.

Careful control of aeration was required during incubation since the amount of frothing increases as the cells multiply and release metabolic biproducts into the medium. Rapid aeration (10 bubbles/second), therefore, was allowed to take place in the first 12 hours of growth, but thereafter a slower rate (1 bubble/second) was maintained.

e) Incubation

Incubation was carried out at 35°C for 48 hours unless growth was weak, in which case the time was extended to 66 hours.

f) Collection of cells

The purity of the batch cultures was examined microscopically by a Gram stain of a smear. On the satisfactory result of the microscopic examination the cultures were subjected to continuous flow centrifugation in a Sharples centrifuge; the sedimented cells were

washed twice in isotonic saline, and then the spun cells were deep frozen (-18°C) in universal containers. The yield from one flask varied from between 5 and 20 ml. of packed cells, depending on the strain cultured. A loopful of spun cells was plated out on to 10% horse blood agar (Dept. of Bacteriology - Immunology, University of Glasgow) to examine the degree of contamination of the cells by other bacteria, since this was unavoidable during centrifugation and washing. The contaminating organisms were always in very small numbers, and since the cells were kept at -18°C until they were disintegrated, the contaminants were not able to grow and multiply, and produce enzymes in large enough amounts to be observable in electrophoretic analysis.

Method 2 - Agar culture of cells

With the arrival of the Braun M.S.K. homogeniser I was able to bring disintegration of packed cells and I found that, on the average, 5 ml. of packed cells gave at least 2 ml. of cell contents, which was quite sufficient for about 50 electrophoretic runs, a number well in excess of my needs. Therefore Method 2 was designed to allow the production of packed cells on a smaller scale and in a more convenient way than Method 1.

It was essential first, however, to ascertain that the enzymic patterns, detectable in polyacrylamide gels, were identical in the cell contents of cells prepared by both Methods 1 and 2. The cell contents of 3 strains, prepared by both Methods were electrophoretically

analysed for esterase (page 203) and blood band isozyme (page 219) patterns. The esterase and blood band isozyme patterns of the cell contents, prepared by the large scale broth culture method (Method 1), and also by the agar culture method (Method 2) were identical, by both methods, for each of the 3 strains.

Since it was shown, therefore, that there was no advantage in preparing cells by Method 1, Method 2 became the method of choice, and I obtained packed cells of 296 different strains of the micrococci and staphylococci by this method.

a) The medium

Several standard laboratory agar media of different chemical composition were examined for their suitability for growing strains of micrococci and staphylococci. Blood Agar Base No. 2 (Oxoid) was the medium of choice.

The medium was sterilised for 15 minutes at 15 lb./sq.in of steam.

About 150 ml. of sterile molten medium was poured on to one side of a Roux bottle, and when this had set, a further 150 ml. of the medium was poured on to the other side. The Roux bottles were then incubated overnight at 37°C to ensure that the medium was still sterile.

b) Inoculation

An inocolum of 5 ml., prepared from an overnight nutrient broth (Oxoid) culture grown at 30°C, of a strain was checked for purity by Gram's stain, and if this was pure the inoculum was added

aseptically to a Roux bottle. The inoculum culture was distributed over the surface of the medium by turning the bottle until all the agar had been covered.

c) Incubation

Inoculated Roux bottles were incubated at the strains' optimum temperature (usually 30°C) for two days, or if growth was poor after this time the bottles were left for a further period of incubation.

d) Collection of cells

Growth was emulsified in distilled water in the Roux bottles with a clean glass rod and the thick cell suspensions were centrifuged at 3000 r.p.m. for 30 minutes. Although the removal of growth from the Roux bottles and the subsequent spinning down were not carried out under strictly aseptic conditions, negligible contamination occurred during these processes. Contamination was judged by microscopic examination of Gram stained smears, prepared from growth of cultures of 10 strains before removal from the Roux bottles and also from the packed cells after spinning down; no contamination was observed microscopically after the centrifugation. Therefore, if smears prepared from growth in the Roux bottles, and stained by Gram's method, showed no contamination when examined microscopically, the spun cells were considered to be sufficiently pure for electrophoretic analysis.

As the Braun M.S.K. homogeniser was available when Method 2 was used for the preparation of cells, there was no need to store the

cells under deep frozen conditions, and they were disintegrated immediately after spinning down.

Disintegration of cells

As the Braun M.S.K. homogeniser was not available at the beginning of this work, various standard methods for disintegrating bacteria i.e. a) autolysis of cells, b) freezing and thawing of cells, c) changes in osmotic pressure, d) Mickle Shaker treatment with glass beads, e) ultrasonic treatment with, and without glass beads, were used to try to break down a selected number of the micrococcal and staphylococcal strains. None of these trial methods were, in fact, successful, and the cells of the 110 strains, cultured by Method 1, had to be stored at -18°C until the homogeniser was available.

The Braun homogeniser disintegration method

Cell suspensions and Ballotini glass beads size no. 12 were mixed to give a thick, but relatively mobile slurry. Disintegration was carried out at 0°C, or below, for 5 minutes at 4000 r.p.m. in special glass Duran Bottles (Shandon Scientific Co. Ltd., London), which fit into the Braun M.S.K. homogeniser. The cooling system requires liquid carbon dioxide, obtainable from Distillers Co. Ltd., Great Britain. Cell suspensions of all 406 strains were broken down by this method.

Antifoam agents

Disintegration with the Braun homogeniser tends to cause frothing of cell suspensions, and this could lead to a denaturation of the

cellular enzymes, and also it was shown that octen-2-ol (B.D.H.) and tributyl-n-citrate (B.D.H.) antifoam agents had no deleterious effect on the enzyme activity of cell contents. These chemicals, therefore, were used to prevent foaming during disintegration (3 drops from a pasteur pipette per cell suspension).

Determination of the percentage cell break-down

Since micrococcal and staphylococcal cells lose the character of Gram positivity when they are broken down it is relatively easy to give an estimate of an approximate percentage of disruption by carrying out a Gram stain on a smear prepared from a disintegrated cell suspension. A minimum break-down of 75% of the cells was required although a higher percentage was preferred. If a lower percentage was estimated the cell suspension was subjected to a further period of disintegration until 75% of the cells or more were disintegrated.

The removal of insoluble particles from the disintegrates

The soluble cell contents of the 406 strains were separated from unbroken cells, cell walls, and Ballotini beads by filtration followed by centrifugation. The following procedure was carried out for the separation.

The removal of Ballotini beads

The glass Ballotini beads were removed from the disintegrates by one of the following methods:-

1) Filtration of the disintegrates through Whatman No. 1 filter

paper with the aid of vacuum pressure. The filter paper retained the glass beads, but allowed cells and cell material to pass through.

2) Filtration of the disintegrates through Hemmings Filters. Pieces of Kleenex tissue, cut out to the correct size, were placed, instead of the usual sterilising Seitz filter pads, into the filters, and the disintegrates were centrifuged at low speeds (2000 r.p.m.) through the filters so that the Ballotini beads alone were retained by the Kleenex tissue. For small quantities of cell material Filtration Method 2 was quicker than Filtration Method 1.

The Ballotini beads were cleaned in chromic acid, washed thoroughly, dried, and used again.

The removal of unbroken cells and cell walls

The unbroken cells and cell walls were separated from the soluble part of the disintegrates by high speed centrifugation (9,000 rp.m.) for 30 minutes.

Storage of cell contents

The soluble cell contents, stored in bijou bottles, were labelled with the appropriate strain numbers, and all preparations were kept at -18°C until required for electrophoretic analysis.

ELECTROPHORESIS OF CELL CONTENTS

Preparation of gel support media for electrophoresis

I decided to use zone electrophoresis in order to detect isozyme bands in cell contents of micrococci and staphylococci, and to find

out if these bands could be used as characters for classifying these organisms. Only 2 gel support media give separation of protein on the basis of both molecular weight and charge during zone electrophoresis - starch and polyacrylamide. Although starch gels have been more frequently used for the detection of isozymes than polyacrylamide gels, the latter have undoubted advantages over the former (page 182). Most of the experimental electrophoretic work in this thesis was carried out with polyacrylamide gels.

It is worth noting here that the recognised nomenclature of gels prepared from "Cyanogum" is not clear; some authors use the term "polyacrylamide", others simply "acrylamide". In this thesis, from now on, I use the shorter term "acrylamide" to describe a gel that has been formed from the polymerisation of acrylamide monomer which is the constituent of commercial "Cyanogum".

Starch gel (Baillie, 1964)

a) Gel medium

Suspensions of 12% hydrolysed starch (Connaught Laboratories, Canada) were prepared in 120 ml. amounts in "tris"-citrate gel buffer, composed of 0.459% $(\frac{W}{V})$ tris (hydroxymethyl)aminomethane and 0.053% $(\frac{W}{V})$ citric acid in distilled water (pH 8.7).

b) Preparation of starch gels

The gel medium was heated in a side-arm pressure flask over a bunsen until the starch turned from an opaque suspension into a clear homogeneous viscous solution, which was subsequently evacuated by

attaching the flask to a pressure pump. This prevented, within the solidifying gel, the formation of bubbles which would distort the flow of the current across the gel during electrophoresis. The molten starch solution was then poured into a glass mould tray ($4\frac{1}{8}$" x $6\frac{1}{8}$" x $\frac{1}{4}$"), the surface was covered with a sheet of "Melinex O" film gauge 50 (Polyester film, I.C.I. Ltd., England) and the gel left until set (2 hours at room temperature).

c) Insertion of the cell contents into the gel

When the gel had set, the "Melinex" cover was removed and the gel was cut across, approximately 3 cm. from one end and the shorter piece of the starch gel slipped gently back to expose the cut surface. Whatman No.1 filter paper was cut into 10 mm. lengths and these were soaked in the cell contents of different strains. After the excess liquid had been removed, by touching the soaked filter strips on absorbent paper, the strips were placed along the cut surface of the starch gel, which was then closed by replacing the short piece of gel. The gel with cell contents was then ready for electrophoretic analysis.

Acrylamide gel (Baillie, 1964)

A Perspex mould was constructed as shown in Diagrams III and IV with an internal volume of 19 x 12.5 x 0.6 c.m. The design of the mould is more advanced than the designs of Murray (1962) and Baillie (1964), in that no clamps or grease are required to form an airtight seal between the lid of the mould and the tray. The mould is similar in

Diagram III

Acrylamide gel mould

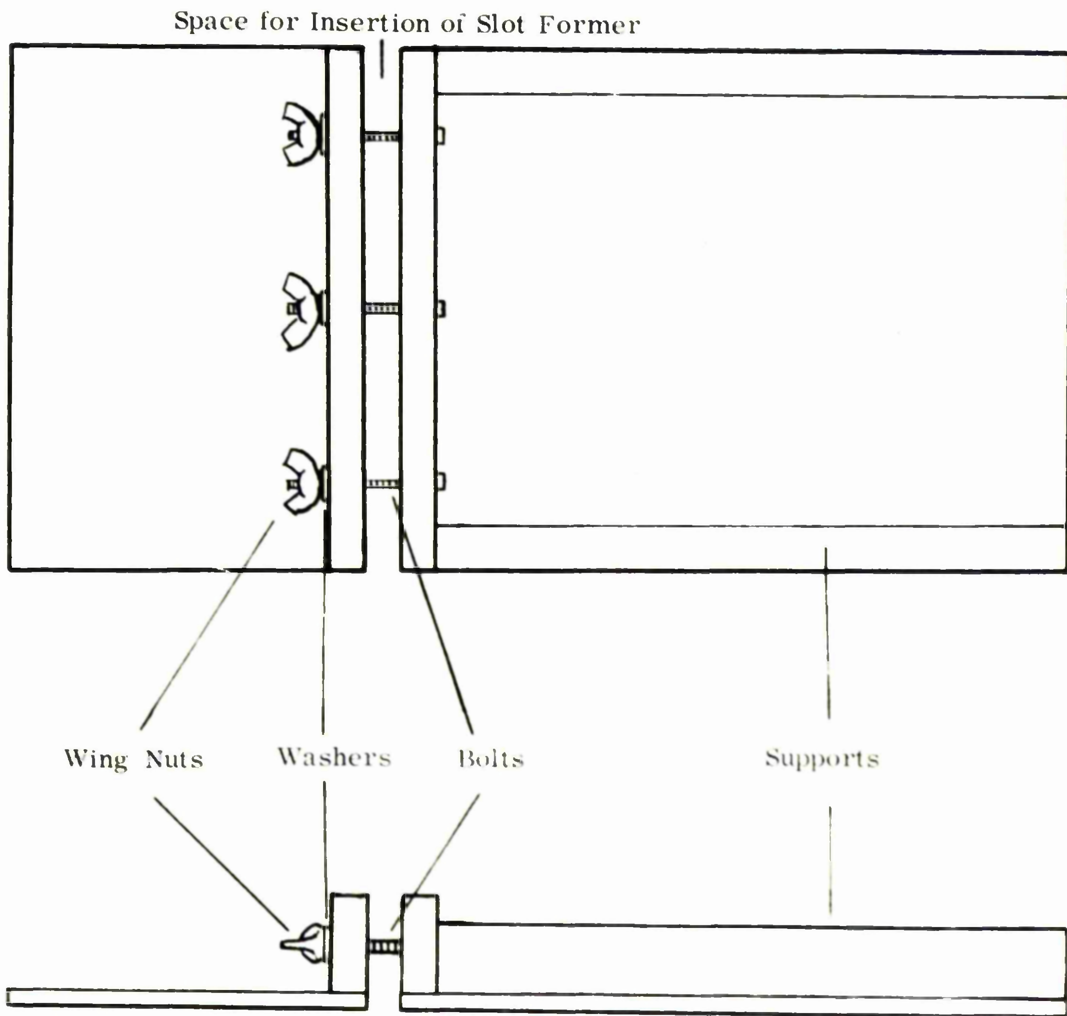
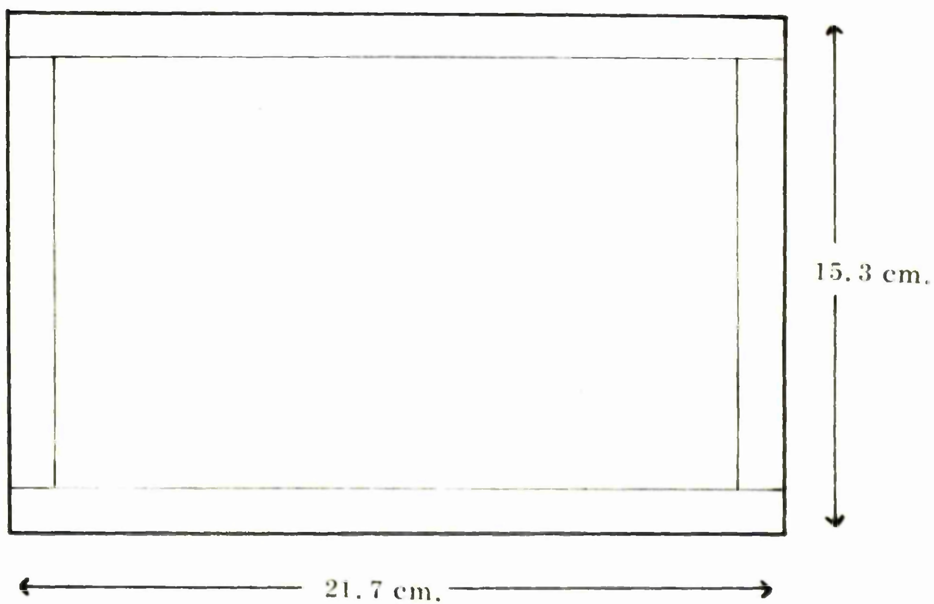


Diagram IV

Acrylamide gel mould

TRAY OF MOULD

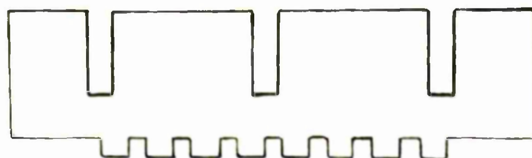
PLAN



ELEVATION

SLOT FORMER

Surface Held to Lid of Mould by Bolts and Nuts



Surface Inserted into Gel

PLAN

design, however, to that of Lund (1965) who incorporated a removable slot former in the lid so that projecting teeth of any number, size, or thickness could be used for the formation of slots. Since the design of her mould is based on that of Smithies (1959) for the formation of starch gels, Lund (1965) did not give a detailed description of its construction, and therefore technical information concerning my mould is given below.

The slot former that I used for the formation of slots in the electrophoretic analysis of the cell contents of the 406 strains was composed of 1 mm. thick Darvic (I.C.I., England), a plastic compound, and was shaped by myself to the design shown in Diagram IV so that 8 slots could be formed in any one gel. A similarly designed slot former was also made for experimental purposes out of Darvic, this time 2mm. thick. The gel mould was constructed in such a way that when the slot former was inserted into the catalysed gel monomer the teeth were suspended 1 mm. above the floor of the tray, so that a thin layer of gel could form below the teeth when the acrylamide polymerised; this was to ensure that the cell contents did not leak out of the bottom of the slots during electrophoresis.

Two gel mould trays were constructed so that two electrophoretic experiments could be carried out simultaneously.

b) Gel medium

Concentrations from 6 to 15% ($\frac{w}{v}$) of Cyanogum 41 (B.D.H.), composed of acrylamide monomer, were prepared in "tris"-citrate buffer of similar

composition to that used in the starch gel (page 196). Acrylamide solutions could be stored at 4°C for 4 weeks without loss of gelling ability. Acrylamide solutions can only be gelled by the presence of catalysts, which polymerise the acrylamide monomer to form NN'-methylenebisacrylamide; two such gelling catalysts, ammonium persulphate and 2-dimethylaminoethyl cyanide, were used together to cause polymerisation. These catalysts were prepared as 10% solutions and could be stored at room temperature for two months without loss of catalytic ability.

c) Method

To 170 ml. of an acrylamide solution, usually at a concentration of 9%, 2 ml. of a 10% solution of each catalyst was added, and the mixture rapidly evacuated in a side-arm pressure flask, since gelling occurs only in the absence of oxygen. When the catalysed solution was completely evacuated it was ready for pouring into the mould.

d) Gelling conditions

After the evacuated catalysed acrylamide solution had been poured into the tray of the mould, the lid, with slot former in position, was carefully lowered on to the tray and left. It was important that no air bubbles were allowed to enter between the solution and the lid as they would have prevented complete polymerisation from taking place. Gelling normally occurred in 30-45 minutes at room temperature, if the mould was not disturbed, but a full hour was allowed to elapse before the lid was carefully removed. The slightly opaque gel in

the tray of the mould was then washed thoroughly in water to remove any toxic monomer or catalyst that might be in free solution.

c) Addition of cell contents

The slots, 9 mm. long, 6 mm. deep 1.5 mm wide and 5 mm. apart, formed in the gel, were blotted dry and molten Paraffinum Molle Album (B.D.H.) was dropped on to the surface area of acrylamide between the slots so that, when samples of cell contents were added to the slots there was no chance of any leakage from one slot into another. When the slots had been filled with the cell contents (about 3 drops with a pasteur pipette) the whole area around the slots, and on the actual slots themselves, were covered with molten, but cool, Paraffinum Molle Album, so that evaporation, or spilling of the contents, did not occur during electrophoresis. The cell contents were then ready to be electrophoresed.

Electrophoresis of cell contents on starch and acrylamide gels

Electrophoresis of cell contents was carried out in a tank (Shandon Scientific Co. Ltd., London), by using a discontinuous buffer system based on that described by Poulik (1957), with the "tris"-citrate gel buffer differing from the tank buffer, which was composed of boric acid 1.85% ($\frac{W}{V}$); NaOH 0.2% ($\frac{W}{V}$) in distilled water (pH 8.1). Contact was made between the tank buffer and the gels by means of absorbent lint wicks. A sheet of "Melinex" covered each gel and the ends of the wicks lying on the gel during electrophoresis. Electrophoresis was carried out at constant voltage from a Vokam

Power Supply (Shandon Scientific Co. Ltd., London) by using one of two methods of time/voltage combination:

Method 1) 50 volts for about 9 hours - acrylamide gels - no appreciable heating up of gel;

Method 2) 150 volts for about 4 hours - starch and acrylamide gels - since gels heat up at this voltage, owing to the resistance, cooling was accomplished by addition of ice directly on to the gels.

Electrophoresis was continued by both methods until a "brown line" artefact, the interface between the "tris"-citrate gel buffer and the borate tank buffer, had travelled 10 cm. from the slots towards the anode. The gels were then removed from the tank, washed free from buffer, and the gel cast from the tray in preparation for slicing.

Slicing of gels

Both starch and acrylamide gels were sliced in the following manner:- Perspex guide strips, to control the thickness of the slices, were placed on either side of each gel, and an empty mould tray placed on top of the gel to maintain an even pressure while slicing. Four horizontal slices, each about 1.5 mm thick, were obtained from a gel which was cut with a fine stainless steel suture, size 36 (London Splint Co., Ltd., England), held taut between the fingers. Only the two middle slices, however, were normally retained for protein or enzyme detection, since trailing of bands may occur along the upper and lower surfaces of the gel. The gel slices were then ready for the detection of protein or enzyme bands.

METHODS FOR DETECTING PROTEIN AND ENZYMES IN GEL SLICES

METHODS FOR DETECTING PROTEIN AND ENZYMES IN GEL SLICES

Many varied methods for detecting protein and enzyme bands in electrophoretically-run slices were attempted, because it was thought that some of these could be used for the classification of micrococci and staphylococci. Protein bands were detected solely by a standard staining reaction. Attempts were made, however, to detect enzymes in gel slices by direct staining reactions, overlay methods and saturation methods. The direct staining reactions are based on those used in histochemical studies for the detection of enzymes in mammalian tissue sections. The methods for detecting enzymes by direct staining are, for convenience, called "enzyme stains" - e.g. esterase stains. The term refers to a coloured compound being formed when the product of the enzymic reaction combines with an indicator or "developer" solution. In some instances a colourless chemical, like a diazonium salt, reacts with the product of hydrolysis to form a coloured insoluble compound; in others, the product of the enzymic reaction releases an insoluble dye which precipitates in situ. The overlay and saturation methods have not been used before to discover the presence of enzymes either in tissue sections or in gel slices. In the overlay methods, an electrophoretically-run gel slice is placed on top of an agar medium containing the appropriate substrate and then both the gel slice and the agar are incubated together; after incubation visible reactions can be observed in the gel slice or the agar medium. In the saturation methods the gel slice is incubated in a liquid substrate medium, so that the substrate can penetrate into the slice and after incubation visible reactions can be observed

in the gel slice. In the detection of micrococcal and staphylococcal enzymes in gels, two standard methods for demonstrating bacterial enzymes in gels were used - Esterase Method 1, the α-naphthyl acetate method, and the Catalase hydrogen peroxide (H_2O_2) Method - all the other staining, or enzyme detection methods described were experimental.

Unless otherwise stated gel slices were incubated at room temperature.

PROTEIN STAIN - Acrylamide slices

Stain: A saturated solution of Naphthalene Black TS (G.Gurr, Ltd., London) in 5% ($\frac{v}{v}$) acetic acid.

This staining solution was poured on to gel slices and allowed to soak in, with help of gentle agitation, for 30 minutes. The background stain was then removed from the slices by successive rinses with 5% acetic acid. Protein bands stained dark blue against the colourless background of the acrylamide.

ESTERASE STAIN

Method 1: the α-naphthyl acetate method (Gomori, 1952b; Lawrence et al., 1960) - Starch and acrylamide slices

Stain: α-naphthyl acetate (1% $\frac{w}{v}$ in 50% acetone), 5 ml.;

Fast Blue B or Fast Blue BB or Fast Blue RR diazonium salts (G.Gurr Ltd., London), 50 mg.

Buffer solution at pH 6.4 containing 0.1M "tris", 60 ml. and 0.1M maleic acid, 40 ml.

Gel slices were flooded with the freshly prepared staining solution and incubated from between 1 and 6 hours with gentle agitation, so that the stain was not deposited unevenly on the gel slices. Esterase activity was shown by the appearance of bands, varying in colour from dark red to black depending on the salt used.

Esterase Method 1 Tube Test

Into a test tube (10 x 0.7 cm.) was placed two drops of "tris"-maleate buffer at pH 5.2, containing 0.5 mg. of Fast Blue B salt, and 2 drops of enzyme solution (composed of the pooled cell contents of four strains, which gave 6 esterase bands of different R_f values by Esterase Method 1). A control tube was set up in the same way, except that the enzyme solution had been previously boiled. Both the test and control solutions were incubated at 30°C for 6 hours.

Method 2: the naphthol AS acetate method (Gomori, 1952b) - Acrylamide slices

Stain: A 1% $\frac{W}{V}$ solution of naphthol AS acetate (G.Gurr Ltd., London) was prepared in equal parts acetone and propylene glycol (B.D.H.) from which 1.0 ml. was added to 15 ml. propylene glycol and the mixture shaken. This solution was then diluted, with water with constant stirring, to form a total volume of 50 ml. To this, 5 ml. of 0.2M phosphate buffer at pH 6.5 was added, followed by 25 mg. of Fast Garnet GBC salt (G.Gurr Ltd., London).

The freshly prepared staining solution was filtered on to gel slices, which were incubated overnight either at 37°C or at room temperature, and then the slices were examined for dark red bands.

Alterations in the pH of the phosphate buffer and the use of "tris"-maleate buffer and other diazo salts were carried out in an attempt to improve the stain for detecting esterase bands.

<u>Modified Esterase Method 2</u>

Esterase Method 2 was altered so that a "tris"-maleate buffer at pH 7 and Red TR salt were substituted for the phosphate buffer and the Garnet GBC salt respectively.

<u>Esterase Method 2 Tube Test</u>

Into each of 4 test tubes (10 x 0.7 cm.) were placed 2 drops of phosphate buffer at the appropriate pH (4.8, 6.0, 6.5 or 6.9), containing about 0.5 mg. of diazo salt (either Garnet GBC or Fast Blue B) and two drops of enzyme solution of the same composition as that used in Esterase Method 1 Tube Test. Duplicate sets of tubes were prepared for controls, except that the enzyme solution had been previously boiled. Both the test and the control solutions were incubated at $30^{\circ}C$ for 6 hours.

<u>Method 3: the indoxyl acetate method</u> (Holt & Withers, 1952) - <u>Acrylamide slices</u>

<u>Stain:</u> 50 mg. of indoxyl acetate (L.Light & Co. Ltd., England) were dissolved in 1.0 ml. ethanol which was then added rapidly with shaking, to a solution containing the following: "tris" - HCl buffer (pH 8.5), 20 mls; oxidising solution containing potassium ferrocyanide (5×10^{-2}M) and potassium ferricyanide (5×10^{-2}M), 10 ml; M calcium chloride, 1 ml; 2M sodium chloride, 50 ml. Distilled water was finally added to bring the volume of the solution up to a total of 100 ml.

Gel slices were incubated in the staining solution overnight either at 37°C or at room temperature and then examined for dark blue bands. Pooled cell contents of 4 micrococcal and staphylococcal strains, giving 6 esterase bands of different R_f values detected by Esterase Method 1, were examined by this method.

The "tris"-HCl buffer was used at various pHs in an attempt to improve the indoxyl acetate stain for the demonstration of esterases.

Method 4: the bromo-indoxyl acetate method (Holt, 1958) - Acrylamide slices

Stain: 13 mg. of 5-bromo-indoxyl acetate (L.Light & Co., England) were dissolved in 1 ml. of ethanol and substituted for the indoxyl acetate in Esterase Method 3; otherwise this method is identical to Method 3.

The same pooled cell contents that were used for Esterase Method 3 were used in Esterase Method 4.

"Tris"-HCl buffer, phosphate buffer, and "tris"-maleate-NaOH buffer were all used at various pHs, and the concentration of other constituents of the bromo-indoxyl acetate stain were altered in attempts to improve Esterase Method 4 for the demonstration of esterases.

ACID PHOSPHATASE STAIN - Acrylamide Slices

Method 1: the metal salt method (Gomori, 1950c;1952b)

Stain: 0.6 g. of $Pb(NO_3)_2$ was dissolved in 500 ml. of 0.05M acetate buffer (pH 5.0) to which was then added 50 ml. of a 3% (0.1M) of sodium glycerophosphate.

After the staining solution had been incubated for 24 hours at 37°C it was filtered and poured over gel slices which were incubated at 37°C or room temperature for up to 24 hours. Sites of phosphatase activity became chalky white from the deposition of lead phosphate. The slices were then rinsed in water, acidified in 1% acetic acid and finally placed in a dilute solution of ammonium sulphide (2 drops $(NH_4)_2S$ in 50 ml. of water) for about 3 minutes, after which sites of activity became dark brown owing to the conversion of lead phosphate to lead sulphide.

Method 2: the α-naphthyl phosphate method

Stain: 50 mg. of sodium α-naphthyl phosphate were added to 50 ml. of distilled water and then this solution mixed with 50 ml. of acetate buffer (pH 5.0). 60 mg. of Fast Red Violet LB salt (G.Gurr, Ltd., London) were dissolved in the solution which was then filtered, and finally 2 drops of a 10% solution of $MnCl_2$ were added before use.

Gel slices were incubated overnight in the staining solution at 37°C or at room temperature, and then they were examined for dark red bands. Pooled cell contents of 4 strains, including one producing a band detected by Phosphatase Method 1, were examined by this method.

The "tris"-HCl buffer was used at pHs varying from 3 to 7, and Red 3GS salt (G.Gurr Ltd., London) was substituted for Fast Red Violet LB salt in attempts to improve the α-naphthyl phosphate stain for the

demonstration of acid phosphatases.

Method 3: the naphthol AS phosphate method

Stain: 25 mg. of naphthol AS AN phosphate (Nutritional Biochemicals Corporation, Cleveland, Ohio) were dissolved in 1.5 ml. of DMF (dimethylformamide; B.D.H.) and this solution added to 100 ml. of "tris"-maleate buffer (pH 6.8) followed by 30 mg of Fast Red TR salt (G.Gurr, Ltd., London).

The gel slices were incubated in the staining solution for 12 hours at either 37°C or at room temperature, after which dark red bands should appear. The same pooled cell contents that were used for Phosphatase Method 2 were used in Phosphatase Method 3.

The "tris"-maleate buffer was used at pHs varying from 5.6 to 7.4 and double strength "tris"-citrate buffer used in the preparation of the gel slices in attempts to improve the naphthol AS phosphate method for the demonstration of acid phosphatases.

β - GLUCURONIDASE STAIN - Acrylamide slices

The post-coupling method (Seligman et al., 1954)

Stain: 30 mg. of 6-Bromo-2-naphthyl-β-D-glucopyranoside (Koch-Light Laboratories Ltd., England) were dissolved in 5 ml. of methanol and then added to a solution containing 20 ml. phospho-citrate buffer (pH 5.0) and 75 ml. distilled water. Gel slices were incubated for 8 hours in this staining solution at 37°C. After this time the slices were rinsed in tap water and immersed in a solution of Fast Blue B (1 mg./ ml.) in phosphate buffer 0.02M (pH 7.5). Slices were incubated for a further 6 hours.

The gel slices were examined for dark brown bands after the final incubation period.

PROTEOLYTIC ENZYME DETECTION - Acrylamide slices

Method 1: aminopeptidase, azo-dye method (Gomori, 1954b; Burstone & Folk, 1956)

Stain: 10 mg. of L-Leucyl- β-naphthylamide hydrochloride was added to a solution containing 8.0 ml. distilled water and 20 ml. of 0.2M "tris"-HCl buffer (pH 7.1), and then 60 mg. of Fast Garnet GBC salt (G.Gurr Ltd., London) were dissolved in the solution.

Gel slices were incubated in the filtered staining solution overnight at 37°C and room temperature, and then examined for dark coloured bands.

The "tris"-HCl buffer was used at pHs 5.7, 7.1 and 7.6 in an attempt to improve the aminopeptidase, azo-dye stain for the demonstration of proteolytic enzyme bands.

Method 2: milk overlay method

Substrate: The basal medium was made up in the following way: 1% ($\frac{W}{V}$) agar (Difco) was dissolved and heated in full strength Ringer's solution. Thiomersalate was added to the cooled, but still molten agar to give a concentration of 1 in 10,000. The medium was stored in approximately 20 ml. amounts in universal containers and these were used when required. The basal medium was renewed every 2 months.

Concentrations of milk, both pasteurised and sterile

skim milk solution, between 1 and 30% ($\frac{v}{v}$) were made up in 50 ml. molten, but cooled, basal medium. This was allowed to set in moulds and the milk agar blocks cast.

The unstained gel slices were placed on the milk agar, both were wrapped up together in "Melinex" to prevent evaporation, and were incubated overnight, and for up to 3 days, at 37°C or at room temperature. The gel slices were then examined for zones of clearing.

Method 3: milk saturation method

Substrate: Pasteurised milk

Gel slices were incubated overnight in the milk at 37°C or 4°C and then examined for zones of clearing.

Method 4: gelatin overlay method

Substrate: Gelatin at concentrations of between 1 and 20% ($\frac{w}{v}$) in water was substituted for milk in Proteolytic Enzyme Detection Method 2.

When the incubation period was complete, both the gel slices and the gelatin overlay were examined for zones of clearing after they had been soaked in acidic mercuric chloride.

Method 5: gelatin saturation method

Substrate: Solutions of gelatin in concentrations of between 1 and 10% ($\frac{w}{v}$).

Gel slices were incubated overnight at 37°C, room temperature, or 4°C in the substrate solutions and then the saturated

gel slices were examined for zones of clearing after the addition of acidic mercuric chloride.

UREASE DETECTION - Acrylamide slices

The urea overlay method

Substrate: 2% ($\frac{w}{v}$) urea and thiomersalate (to give a final concentration of $\frac{1}{10,000}$) in various molten but cooled urea basal media. The urea agar media were poured into moulds and cast as blocks when set.

Basal Medium 1 - Christensen's (1946) urea basal medium, including phenol red as indicator (pH 7).

Basal Medium 2 - Christensen's (1946) urea basal medium including phenol red as indicator, buffered with "tris"-HCl to give pHs of 5 and 6.

Basal Medium 3 - 1.5% ($\frac{w}{v}$) agar (Difco) containing 25 mg./100 ml. thymol blue indicator (B.D.H.) buffered with "tris"-HCl to give pH of 6.

Basal Medium 4 - Basal Medium 3 containing ½ strength Ringer's solution.

Basal Medium 5 - Basal Medium 3 with an increased concentration of indicator 50 mg./100 ml.

Each gel slice was laid over the cast urea agar blocks, and both were incubated together as for Proteolytic Enzyme Detection Method 2 (page 209). Urea bands were recognised by colour changes in the pH indicators, occurring in discrete zones, in both the gel slices and

the urea overlay blocks. Results were normally read from the gel slices.

The urea tube method

Strains were grown overnight on Blood Agar Base No. 2 slopes at 30°C, the growth suspended in sterile distilled water (about 0.5 - 1 ml.) to give a creamy consistency, and the suspensions added to a test tube containing 1.5 ml. of a urea medium (page 223). These tubes were incubated at 30°C for 30 minutes and the contents examined for the production of ammonia, indicated by a colour change in the pH indicator.

The urease induction method

Strains were grown in Blood Agar Base No. 2 containing 2% urea for 2 days at 30°C and then the cells suspended in sterile distilled water and tested by the urea tube method (page 212).

LIPASE DETECTION - Acrylamide slices

Method 1: the 2-naphthyl laurate method

Stain: 2-naphthyl laurate (Koch-Light Laboratories Ltd., England) was substituted for α-naphthyl acetate in Esterase Method 1 (page 203).

Gel slices were incubated overnight either at 37°C or at room temperature and then examined for dark bands.

Method 2: the tributyrin overlay method

There are two slightly different approaches to this overlay method.

a) Molten Tributyrin Agar (Oxoid, page 112) was poured into moulds and allowed to set. Gel slices were placed on the surface of the case Tributyrin Agar blocks.

b) Cooled but molten Tributyrin Agar was poured directly on top of gel slices. The agar was kept from solidifying by maintaining the temperature at 56°C for 10 minutes; this allowed the Tributyrin Agar to soak in to the slices. The agar medium within and on top of the slices was cooled so that it set in situ.

The gel slices and the substrate medium were wrapped up in "Melinex" to prevent evaporation and incubated at 37°C, room temperature, or 4°C. Areas of clearing in the opaque Tributyrin Agar appeared after 24 hours, if tributyrinase enzymes were present in gel slices.

Method 3: the Nile Blue sulphate tributyrin overlay method

Substrate: 25 mg. of Nile Blue sulphate (G.Gurr Ltd., London) were first of all added to 100 ml. of the molten Tributyrin Agar, and then this mixture used according to Lipase Methods 2a) and 2b), in place of Tributyrin Agar alone.

Sites of lipase activity could be indicated by blue zones in the gel slices.

Method 4: the tributyrin saturation method

Substrate: 25 mg. of Nile Blue sulphate in 100 ml. of liquid glycerol tributyrate (B.D.H.)

Gel slices were incubated in the substrate medium overnight at 37°C,

room temperature, or 4°C. Sites of lipase activity should show as blue zones.

SUCCINIC DEHYDROGENASE STAIN - Acrylamide slices

Method 1: the Nitro BT method (Nachlas *et al.*, 1957)

Stain: 50 ml. of a buffered succinate solution (25 ml. of phosphate buffer 0.2M pH 7.6; 25 ml. of sodium succinate 0.2M) were added to 50 ml. of an aqueous solution containing 50 mg. of Nitro BT tetrazolium salt (Dajac Laboratories, Philadelphia, Pennsylvania), previously dissolved in 1 ml. of ethanol.

Gel slices were incubated overnight at 37°C, or room temperature, and then examined for blue formazan deposits at sites of dehydrogenase activity.

Method 2: the modified Nitro BT method (Nachlas *et al.*, 1957; modified by Burstone, 1962)

Stain: 40 ml. of distilled water were added, with constant agitation, to 10 mg. of Nitro BT tetrazolium salt previously dissolved in 0.5 of ethanol; 1 mg. of sodium succinate was dissolved in 20 ml. of distilled water and added to the above solution, followed by 40 ml. of 0.2M "tris"-HCl buffer pH 7.4 to bring the total volume up to 100 ml.

Gel slices were incubated in this staining solution overnight at 37°C, and then examined for blue formazen deposits.

LACTIC DEHYDROGENASE STAIN - Acrylamide slices

The Nitro BT method (Dewey & Conklin, 1960b)

Stain: The staining solution was prepared in 100 ml. amounts as follows:- sodium lactate 0.1M; NAD (nicotinamide adenine dinucleotide;

Koch-Light Laboratories, Ltd., England), 0.3mg/ml; PMS (N-methyl-phenazonium methosulphate; L.Light & Co.Ltd., England, 0.02 mg/ml; Nitro BT tetrazolium salt, 0.5 mg/ml; sodium cyanide 0.1M; phosphate buffer 0.25M, pH 7.4.

Gel slices were incubated in this solution for 2 hours at 37°C. Bands showed up as blue formazan deposits.

<u>MALIC DEHYDROGENASE STAIN</u> - <u>Acrylamide slices</u>

<u>The Nitro BT method</u> (Thorne <u>et al.</u>, 1963)

<u>Stain:</u> The staining solution was prepared in 100 ml. amounts as follows:-

sodium L-malate, 0.11M; NAD, 0.7 mg/ml;

PMS, 0.02 mg/ml; Nitro BT tetrazolium salt, 0.4 mg/ml;

0.1M "tris" - HCl buffer pH 8.4.

Gel slices were incubated in this solution for 4 hours to overnight at 37°C. Bands showed up as blue formazan deposits.

<u>PEROXIDASE STAIN</u> - <u>Acrylamide slices</u>

<u>Method 1: the "Nadi" method</u> (a modification of that of Lillie, 1954)

<u>Stain:</u> Two stock solutions were prepared:-

a) α-naphthol (5.76g.) dissolved in 60 ml. of N NaOH and diluted with distilled water to 500 ml.

b) diethyl-p-phenylenediamine monohydrochloride (6.9 g.) dissolved in 500 ml. distilled water.

When 70 ml. of a) and 30 ml. of b) are mixed a dark blue-green colour with a faint white precipitate results (pH 9 - 10).

Gel slices were incubated in this mixture for 1 to 2 hours during which time blue bands appear on the slices.

This method differs from that of Lillie (1954) in that diethyl-p-phenylenediamine monohydrochloride is used instead of the dimethyl compound and also in that 70% of a) is mixed with 30% of b) instead of a) and b) being mixed in equal proportions.

Method 2: the o-dianisidine method (Smith, 1960)

Stain: The following staining solution was prepared:-

1.9M acetate acid buffer pH 4.6, 35 ml; distilled water 80 ml; o-dianisidine (B.D.H.), 1mg/ml. in ethanol 35 ml; H_2O_2, 20 volumes % (added immediately before use), 5 ml.

Gel slices were incubated in this solution for 2 to 3 hours at 37°C or overnight at room temperature, and then they were examined for brown bands.

CATALASE DETECTION - Starch and Acrylamide slices

The H_2O_2 method (Paul & Fottrell, 1961)

Stain: By the nature of this method the presence of starch is required in the gel slices; acrylamide slices were soaked in a cooled dilute boiled starch solution overnight at 4°C and then the slices were rinsed for about 30 minutes in cold tap water to remove excess starch immediately before band detection was attempted.

Gel slices were flooded with 1% ($\frac{V}{V}$) H_2O_2 (100 volumes) for a few seconds and washed in running water to remove excess peroxide. Slices were then placed in 2% ($\frac{W}{V}$) potassium iodine solution acidified with a few drops of glacial acetic acid and incubated for a few minutes.

Catalase bands appeared as clear areas against the dark blue background of the gel slices which had been stained with the iodine released from potassium iodine by the peroxide.

CYTOCHROME OXIDASE STAIN - Acrylamide slices

Method 1: the p-aminodiphenylamine method (Burstone 1959a, 1960a, 1961)

Stain: the incubating solution was prepared as follows:

30 mg. of p-aminodiphenylamine and 30 mg. of 1-phenyl-3-methyl-5-pyrazolone, previously dissolved in 1 ml. of ethanol, were added to 70 ml. of distilled water, which results in a cloudy solution. 30 ml. of 0.2M "tris" - HCl buffer (pH 7.4) were mixed in the above solution and the resultant liquid filtered before use.

Gel slices were incubated in the staining solution at 37°C for 2 hours and then the enzyme bands were made visible by incubating the slices for a further period overnight in 0.003M $Pb(NO_3)_2$ in order to chelate the dye. Bands appeared pinkish-brown on examination of the gel slices.

Method 2: the cytochrome oxidase tetrazolium method (Oda et al., 1958)

Stain: the incubating solution was prepared as follows in 100 ml. amounts:

equal volumes of phosphate buffer (0.1M, pH 7.6), p-phenylenediamine solution in distilled water (0.2M), neotetrazolium chloride solution in distilled water (0.2%) and cytochrome C solution in distilled water (0.0001M) were mixed just before use.

Gel slices were incubated in this solution for 18 hours at 37°C and then examined for dark brown bands.

CYTOCHROME OXIDASE STAIN - Acrylamide slices

Method 1: the p-aminodiphenylamine method (Burstone 1959a, 1960a, 1961)

Stain: the incubating solution was prepared as follows:

30 mg. of p-aminodiphenylamine and 30 mg. of 1-phenyl-3-methyl-5-pyrazolone, previously dissolved in 1 ml. of ethanol, were added to 70 ml. of distilled water, which results in a cloudy solution. 30 ml. of 0.2M "tris" - HCl buffer (pH 7.4) were mixed in the above solution and the resultant liquid filtered before use.

Gel slices were incubated in the staining solution at 37°C for 2 hours and then the enzyme bands were made visible by incubating the slices for a further period overnight in 0.003M $Pb(NO_3)_2$ in order to chelate the dye. Bands appeared pinkish-brown on examination of the gel slices.

Method 2: the cytochrome oxidase tetrazolium method (Oda et al., 1958)

Stain: the incubating solution was prepared as follows in 100 ml. amounts:

equal volumes of phosphate buffer (0.1M, pH 7.6), p-phenylenediamine solution in distilled water (0.2M), neotetrazolium chloride solution in distilled water (0.2%) and cytochrome C solution in distilled water (0.0001M) were mixed just before use.

Gel slices were incubated in this solution for 18 hours at 37°C and then examined for dark brown bands.

MONAMINE OXIDASE STAIN - Acrylamide slices

The tetrazolium method (Glenner et al., 1957)

Stain: The incubating solution was prepared as follows:-

tryptamine hydrochloride (L.Light & Co.Ltd., England) 100 mg.; sodium sulphate, 16 mg.; Nitro BT tetrazolium salt, 20 mg.; 0.1M phosphate buffer (pH 7.6) 20 ml.; distilled water 60 ml.

Gel slices were incubated in this solution overnight at 37°C or at room temperature. Blue formazan deposits should indicate monamine oxidase activity.

DOPA OXIDASE STAIN - Acrylamide slices

The DOPA method (Gomori, 1952b, modified by Burstone, 1962)

Stain: The staining solution was prepared as follows:-

100 mg. of dihydroxyphenylalanine (DOPA) were dissolved in 0.1M phosphate buffer (pH 7.3 - 7.5).

Gel slices were incubated in this solution overnight and longer at 37°C or room temperature. A black pigment, melanin, should appear round sites of DOPA oxidase activity.

STARCH HYDROLYSING ENZYME DETECTION - Starch and acrylamide slices

The H_2O_2 method (Paul & Fottrell, 1961)

Detection: Starch gel slices and starch-soaked acrylamide gel slices were incubated overnight or longer at 37°C, room temperature of 4°C and the method for detecting starch hydrolysing enzyme bands is identical to the method for detecting catalase bands (page 216).

The starch hydrolysing enzyme bands take a few minutes longer to appear than the catalase bands and are yellow-purple at first, brightening to yellow, or even red in some cases, about 30 minutes after the addition of the potassium iodide to the gel slices. Unlike the catalase bands these starch hydrolysing enzyme bands remain visible for many days when they eventually become colourless and only disappear when the contrasting dark blue background of the gel slices has faded, and also they are not seen in starch gel slices.

BLOOD BAND DETECTION - Starch and acrylamide slices

Basal Medium: The basal medium was made up as for Proteolytic Enzyme Detection Method 2 (page 209)

Blood: Whole defibrinated horse blood (Burroughs Wellcome); sheep blood in Alsever solution (Burroughs Wellcome); time expired citrated whole human blood (Department of Bacteriology-Immunology, University of Glasgow); citrated whole rabbit blood (direct from animal's ear).

Detection: 10% ($\frac{v}{v}$) of one of the species of blood, except sheep, was added to 40 ml. of molten basal medium and thoroughly mixed. Sheep blood had to be added to the basal medium at a concentration of 20% ($\frac{w}{v}$) to give the same intensity of red colour as the other bloods in the basal medium. The blood agar was then carefully poured into a Perspex tray (14 x 12.5 x 0.5 cm.) and was allowed to set, after which it was transferred to a film of "Melinex" by inverting the mould and easing the blood agar sheet on to the film. Any drops of

moisture were removed from the agar surface by blotting with fluffless paper (Kleenex). A gel slice was placed on top of the agar sheet, taking care to exclude air bubbles, and the two wrapped together within the "Melinex" film. The package was then placed on a glass plate and incubated at 37°C overnight.

When the gel slice was stripped from the blood agar next morning, the red cells in the blood agar basal medium had undergone partial general haemolysis and the formerly transparent acrylamide had now an overall reddish opacity within which could be seen transparent bands. These bands were made more obvious by soaking the gel slices in water for 12 hours so that the soluble blood components could diffuse out leaving a white opaque background with well defined clear zones. In starch gel slices no reddish opacity or transparent bands were ever seen.

THE EFFECT OF HEAT ON ISOZYMES

Esterase Heating Experiment

Pooled cell contents of 4 micrococcal and staphylococcal strains, each strain possessing different isozyme patterns, were heated in ampoules in a water bath at temperatures that did not fluctuate more

that $\pm$ 0.5°C. Cell contents were subjected to temperatures between 45°C and 100°C for varying periods of time. Gels were run with the heated samples and an unheated control in the slots. After electrophoresis, the gel slices were stained by Esterase Method 1 (page 203), and the slices were examined for loss of esterase activity as far as numbers and strength of bands were concerned.

Peroxidase, Catalase and Blood band Heating Experiment

The same pooled cell contents and the same method were used as for Esterase Heating Experiment (page 220) except that the effect of heat was examined on peroxidase, catalase and blood band isozyme activity, and the cell contents were subjected to temperatures between 45°C and 109°C for varying periods of time.

Succinic dehydrogenase, Monamine oxidase Heating Experiment

The same pooled cell contents and the same method were used as for Esterase Heating Experiment (page 220) except that the effect of heat was examined on opaque bands detected by Succinic Dehydrogenase Method 1 and the Monamine Oxidase Stain, and the cell contents were subjected to temperatures between 45°C and 109°C for varying periods of time.

THE EFFECT OF CHEMICAL INHIBITORS ON ISOZYMES

Inhibition of enzymes in gel slices was attempted by pre-incubating gel slices in a certain concentration of inhibitor and then incubating the slices with specific enzyme detecting system containing the same concentration of inhibitor.

The following chemicals were used to inhibit enzyme activity in acrylamide gels:

Esterases

DPF-Di-iso-propyl fluorophosphate (L.Light & Co.Ltd., England)

Eserine (BDH)

E 600 - Diethyl p-nitrophenyl phosphate (L.Light & Co. Ltd., England)

PCMB - p-chloromercuribenzoate (Koch-Light Laboratories Ltd., England)

3-phenylpropionic acid (Hopkin & Williams Ltd., England)

Peroxidases, catalases and blood bands

potassium cyanide

Succinic dehydrogenases

sodium malonate

Monamine oxidases

potassium cyanide

urea

PCMB

Starch hydrolysing enzymes

mercuric chloride

PHOTOGRAPHY

Diagrams and Scheme 2

The diagrams and Scheme 2 were photographed with a half plate or quarter plate M.P.P. camera on to Kodalith Ortho film (Kodak), and prints were made on Kodagraph Projection paper (Kodak) or bromide air mail photographic paper (Kodak).

Gels

All gel slices were photographed by transmitted light, and acrylamide gels displaying blood bands were taken against a black background, whereas all other gel preparations were taken against a white background. The gels were photographed with an Exa 35 mm. camera on to Microneg Pan 35 mm. (Ilford) and Pan F 35 mm. (Ilford) film, and were printed on bromide air mail photographic paper (Kodak).

One colour plate was prepared from a colour transparency (Kodachrome, Kodak) taken with the Exa camera.

APPENDIX

Urea tube medium

% ($\frac{w}{v}$) in distilled water

KH_2PO_4	0.2
Na Cl	0.5
glucose	0.1
urea	2
phenol red	0.0012

SECTION B

ELECTROPHORETIC ANALYSIS OF CELL CONTENTS OF STRAINS OF MICROCOCCI AND STAPHYLOCOCCI

RESULTS

THE RESULTS OF THE ELECTROPHORETIC ANALYSIS OF CELL CONTENTS OF 406 STRAINS OF MICROCOCCI AND STAPHYLOCOCCI

In the following section I have recorded the results of my electrophoretic analysis of the 406 strains. Many of the experimental methods, especially those based on histochemical stains for mammalian tissue sections, for detecting micrococcal and staphylococcal isozymes were not successful, but I was able to detect the following isozymes with methods giving reproducible results:-

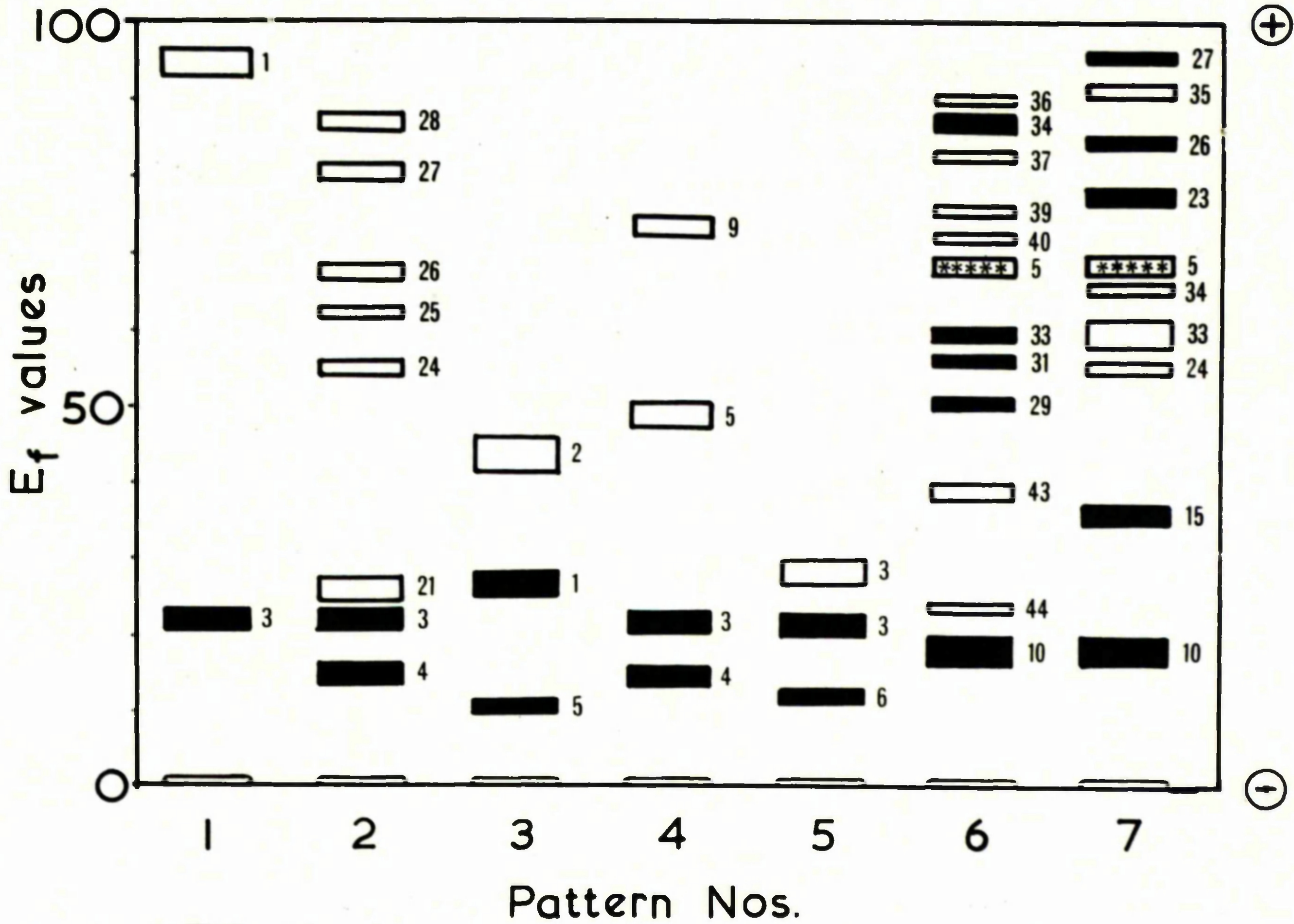
1) esterase isozymes - the α-naphthyl acetate method (page 203).
2) starch hydrolysing isozymes - the H_2O_2 method (page 218).
3) blood band isozymes - the Blood Band Detection Method (page 219).

For each of these enzyme systems, band numbers were assigned to each isozyme. The band numbers (Diagram V) do not correspond to the actual E_f values (electrophoretic mobilities) of the isozymes, although each band number represents a different E_f value. Bands which were present in many strains, were determined first and consequently were given low band numbers; bands which were infrequent and found only in one or two strains, naturally tended to be noticed later and higher numbers were assigned to them.

To ensure that band numbers were correctly assigned, gels were always run with the cell contents of 1 or 2 strains with known isozyme patterns along with the cell contents of those with unknown

Diagram V

Esterase, blood band and starch hydrolysing isozyme patterns produced by strains of some of the commonly occurring micrococci and staphylococci



= Esterase bands

= Blood bands

**** = Starch bands

Isozyme band numbers are marked beside the appropriate bands. Each pattern is representative of an Electrophoretic Group (Scheme **2**) although not necessarily produced by any one strain.

Pattern 1 = Electrophoretic Group 1, containing all the coagulase positive staphylococci.

Pattern 2 = Electrophoretic Group 3, containing many biochemically active staphylococci.

Pattern 3 = Electrophoretic Group 6, containing most of the Staphylococcus epidermidis strains.

Pattern 4 = Electrophoretic Group 9, containing staphylococci found on pig and human skin.

Pattern 5 = Electrophoretic Group 12, containing strains, mostly micrococci from many varied habitats but rarely from the human body.

Pattern 6 = Electrophoretic Group 24, containing all Micrococcus luteus strains.

Pattern 7 = Electrophoretic Group 27, containing all Micrococcus roseus strains.

patterns. In this way it was possible to decide whether a strain possessed isozymes of certain band numbers or not. The cell contents of a strain usually had to electrophoresed many times, each time with a different selection of strains, before all of its isozyme bands were shown to have either the same E_f values as those of other strains, and consequently could be given the same band numbers, or different E_f values, not known to occur in any other strain of micrococci or staphylococci, and new band numbers were assigned to them. Very weak isozyme bands were only given band numbers if they were reproducible. Strains were thus characterised for the 3 enzyme systems.

THE DETECTION OF PROTEIN AND ENZYME BANDS IN GEL SLICES

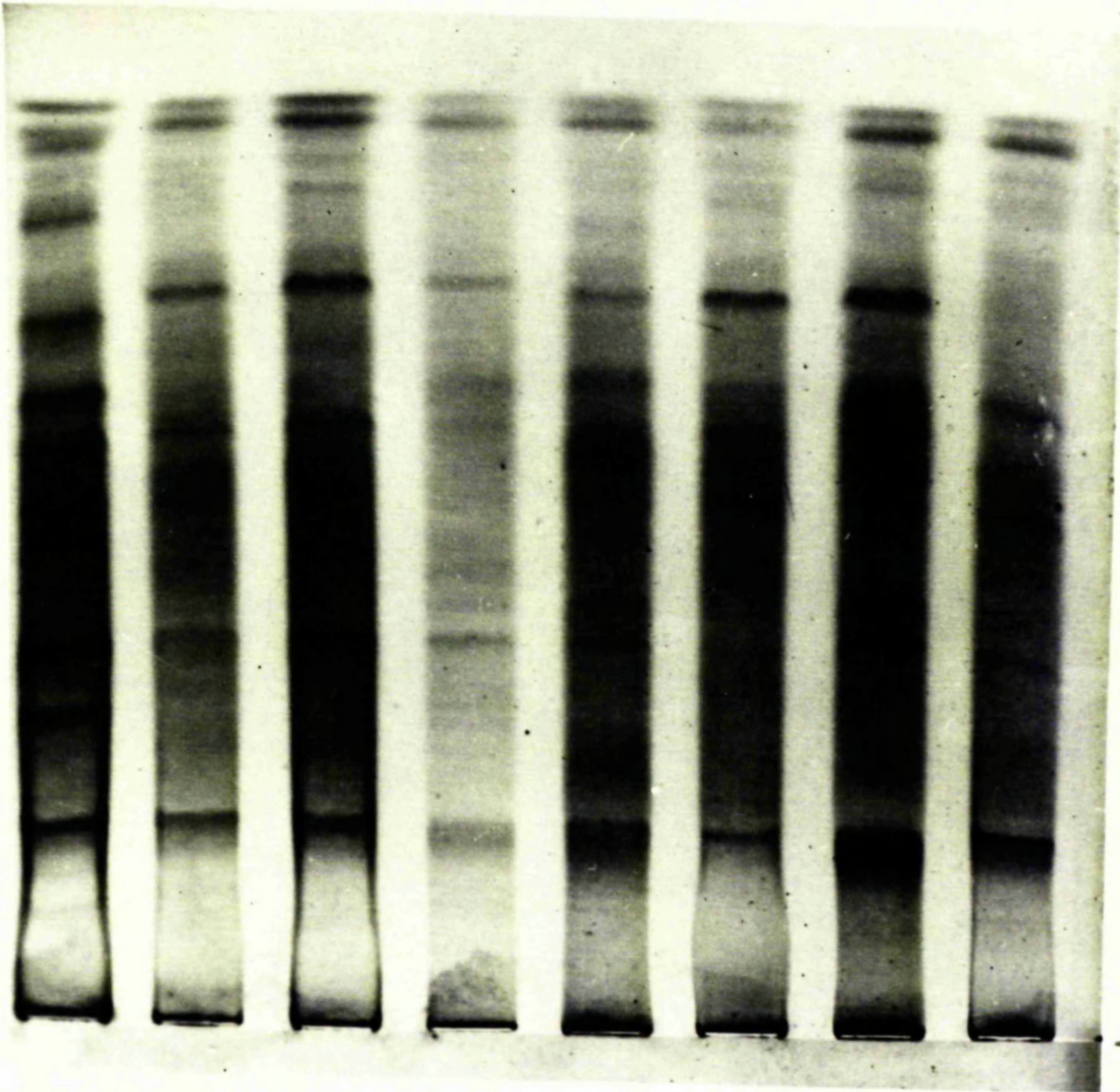
PROTEINS

Protein bands were readily detected in gel slices by the Protein Stain (page 203). So many bands were visible (over 20 in some strains) for each of the 40 randomly selected strains of micrococci and staphylococci examined, however, that it would be almost impossible to compare bands for their electrophoretic mobilities (E_f values) in over 400 strains (Plate 1).

Therefore, I did not use the Protein Stain for the classification of micrococci and staphylococci, but the Stain proved useful for demonstrating that bands stained by certain enzyme detecting methods were, in fact, protein (pages 256 and 261).

Plate 1

Acrylamide gel slices stained by Protein Stain showing protein bands



Electrophoresed cell contents of 8 *Micrococcus roseus* strains

ESTERASE ISOZYMES

Method 1: the α-naphthyl acetate method (page 203)

This method is now recognised as the standard one for demonstrating the presence of bacterial esterases in gel slices (page 182). I found, however, that Fast Blue B salt, the one most frequently used in this method, tended to give a greater non-specific background stain than Fast Blue BB or RR salts, and I preferred to use either of the last two salts in Esterase Method 1.

The cell contents of all 406 strains of micrococci and staphylococci were examined for esterase bands in acrylamide gel slices stained by this method. The total number of esterase bands with different esterase E_f values, detectable by this method, for the 406 strains was 87, and the number of bands recorded per strain varied from 0 (54 strains) to 6 (15 strains) with most strains having 1 or 2 bands. Plates 2, 3, 4 and 10 show esterase bands of selected strains. Diagram V shows patterns of esterase bands typical of certain groups of strains; esterase band numbers are also shown. Esterase isozymes were used in the electrophoretic classification scheme (page 277).

Method 2: the naphthol AS acetate method (page 204)

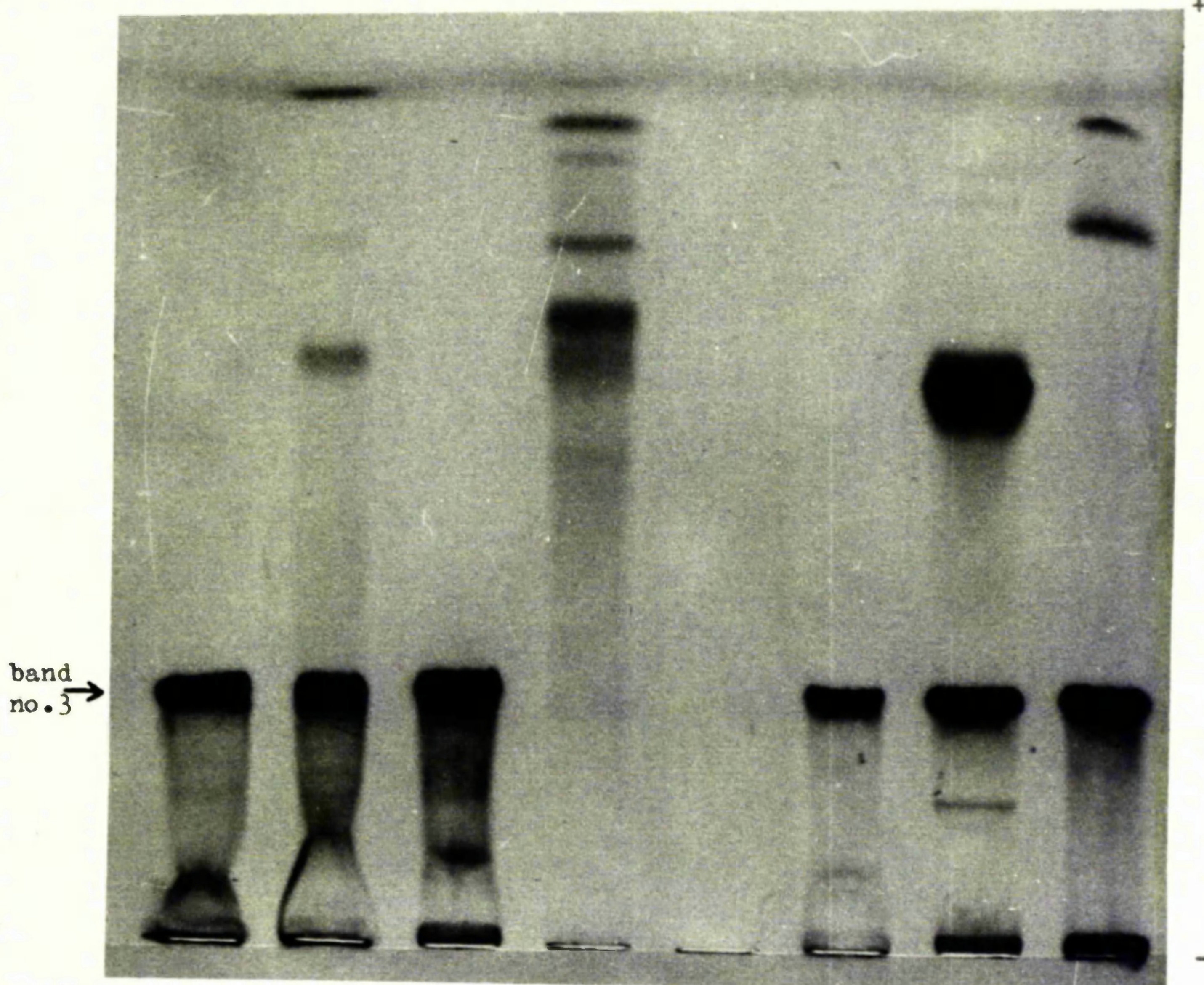
No esterase bands were detected by this method, no matter whether gel slices were incubated in the staining solution at 37°C or at room temperature.

The next stage was to see if any enzymic activity could be detected

Plate 2

Acrylamide gel slice stained by

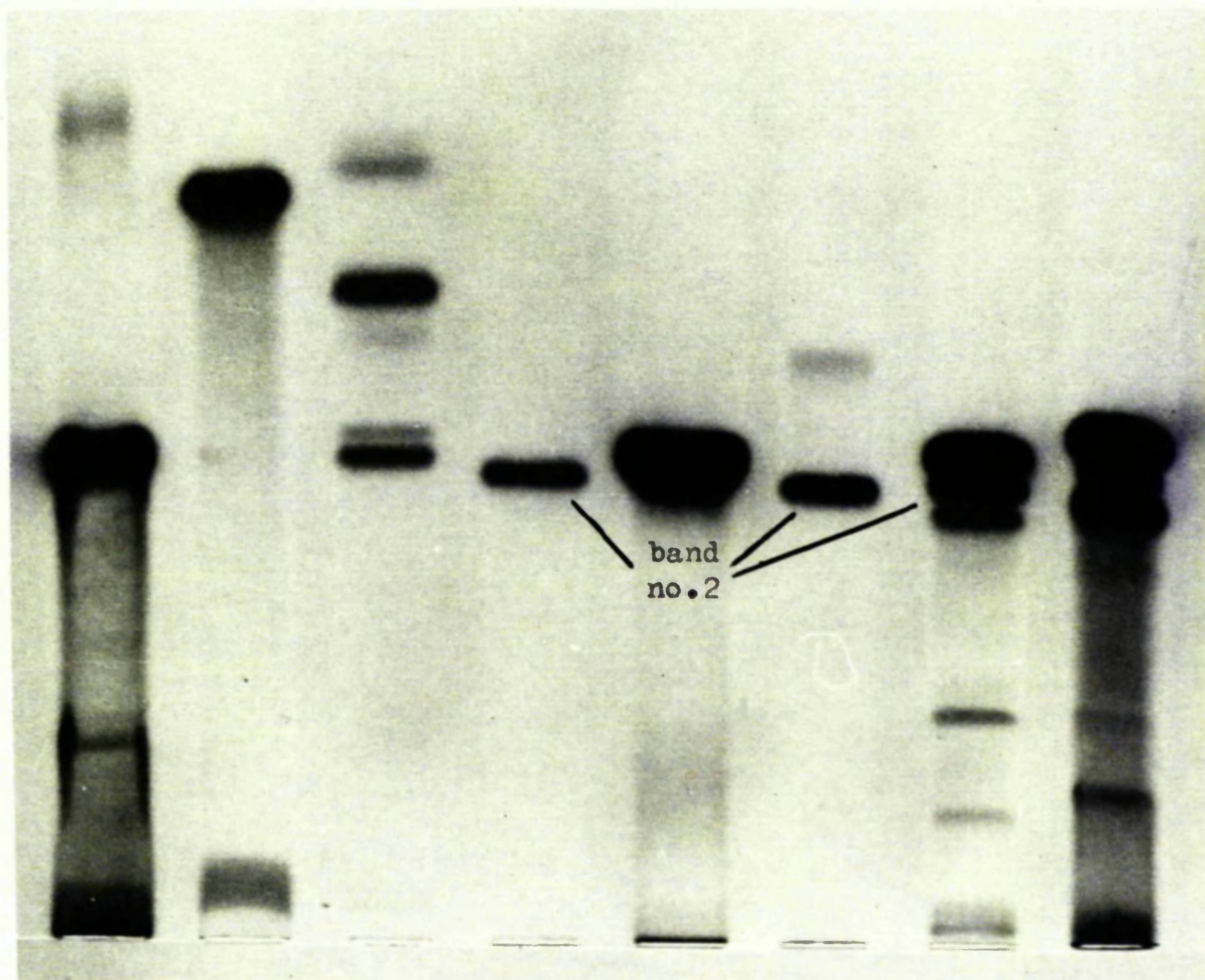
Esterase Method 1 showing esterase isozyme bands



Electrophoresed cell contents of 8 strains isolated from cheeses. The strong low running esterase band is typical of many micrococci found in cheeses.

Plate 3

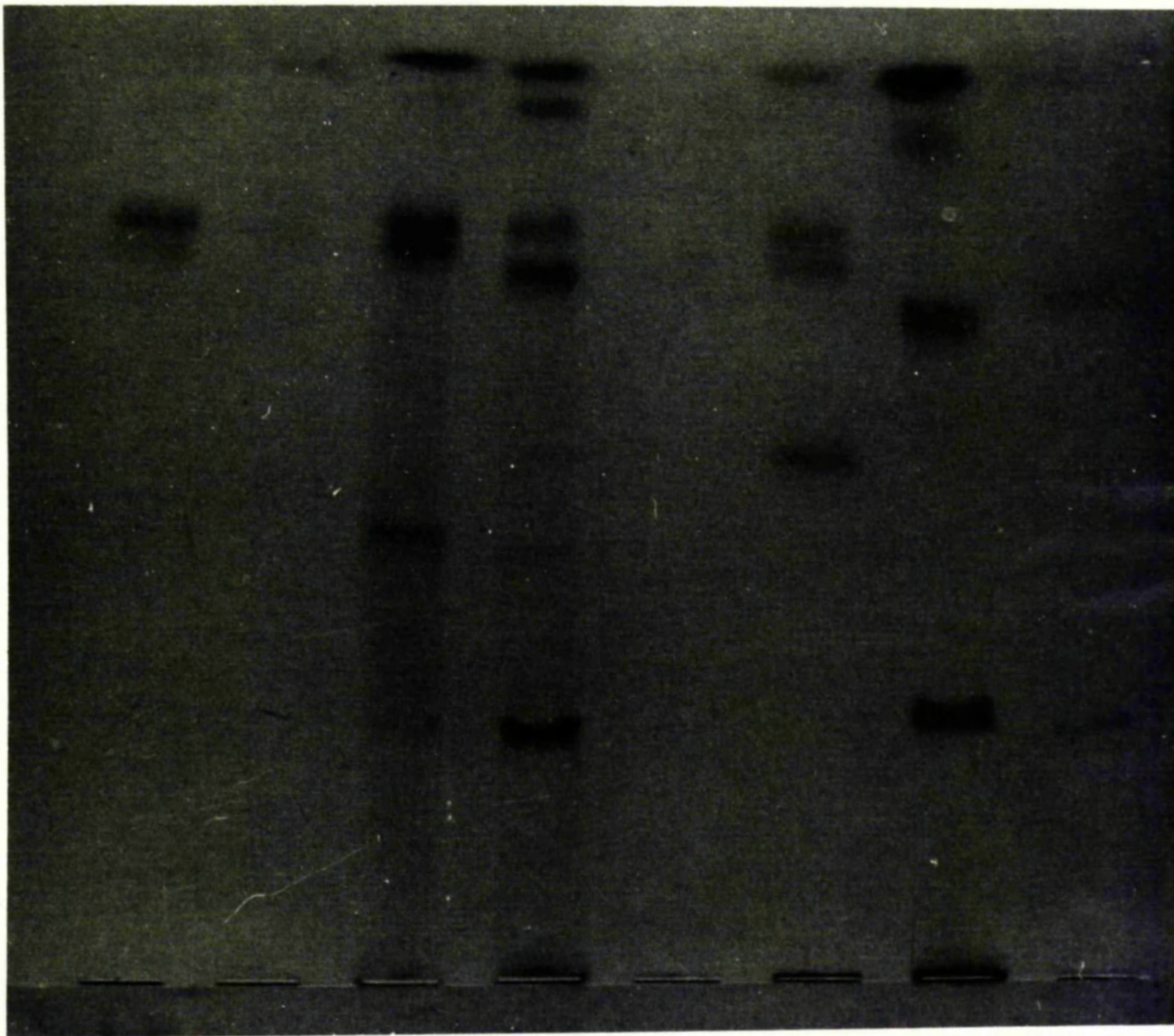
Acrylamide gel slice stained by Esterase Method 1 showing esterase isozyme bands



Electrophoresed cell contents of 8 strains isolated from human and animal skin. This plate shows a great variety of esterase patterns; esterase band no. 2, however, is produced by the majority of coagulase negative staphylococci found on human skin.

Plate 4

Acrylamide gel slice stained by
Esterase Method 1 showing esterase isozyme bands



Electrophoresed cell contents of 8 Micrococcus luteus strains. Micrococcus luteus strains usually produce weak bands, and not all of these show up in photographs. Nevertheless, this plate shows that these strains produce different but related patterns of esterase bands.

by this method on a larger scale in test tubes, and Esterase Method 2 Tube Test (page 205) was carried out. Although the greatest precipitation of insoluble dye should be produced in the test solution at the optimum pH for esterase activity, and also for the coupling of the diazo salt with the released naphthol AS compound (pH 6.5 in human tissue sections), this was not the case in the test tube experiment. In fact, slight precipitation occurred for both diazo salts in the test solution at pH 4.8, and in addition, heavy precipitation occurred at all pHs (although the greatest was at pH 4.8) in the control solutions. These anomalous results could not be explained, and it was decided to re-examine the effect of esterase action in the tube by using a staining method that was known to produce visible esterase bands in gel slices - Esterase Method 1 and Esterase Method 1 Tube Test (page 204) was carried out. Both test and control tubes, however, showed an equal amount of orange dye which had precipitated, and it was concluded that boiled denatured protein could stimulate the production of a precipitate as much as an active enzyme preparation.

It seems that these test tube experiments, where unknown non-specific reactions occurred, did not reflect the mechanism of esterase action and diazo coupling as they took place in the gel slices, and, therefore, they were not used further for attempting to find the correct buffer pH - diazo salt combination for enzyme detection in gel slices.

The final stage in trying to demonstrate enzyme activity with

Esterase Method 2 was to return to experiments with the gel slices, and attempt to detect the presence of bands by altering the pH of the phosphate buffer, and the type of diazo salt, since pH affects the combination of the salt with the released naphthol. The following combinations of salts and buffer pH were used in Esterase Method 2 for demonstrating bands:

Diazo salt	Phosphate buffer pH
Fast Blue B	5.0
Garnet GBC	6.0
Garnet GBC	6.5
Red TR	6.5
Combination of all three salts	6.5

No bands however were detected.

Although Gomori (1952b) used this staining method (Esterase Method 2) with a phosphate buffer of pH 6.5, Pearse (1960; page 887) stated that naphthol AS is particularly sensitive to pH changes when coupling with diazonium salts and the best results are obtained within the pH range of 6.8 - 7.2. Accordingly Modified Esterase Method 2 (page 205) was used to try and demonstrate esterase bands. When this modified stain was used a faint high running band was seen on a gel slice. This band had a different E_f value from any of those detected by Esterase Method 1, for the same cell contents. This anomalous result could be explained by the view of Gomori (1952b),

who thought that the esterase enzymes that act upon naphthol AS acetates differ from the ones that hydrolyse α-naphthyl acetate or indoxyl acetates, at least in human tissue sections.

Even Modified Esterase Method 2, however, did not give such clear patterns with such a wide range of E_f values as Esterase Method 1 and it was decided not to use Esterase Method 2 or its modification for classification purposes.

Method 3 - the indoxyl acetate method (page 205)

No esterase bands were seen in gel slices, even with the "tris"-HCl buffer at pHs 7.0, 7.8, 8.1, 8.5 and 9.3, and no further attempt was made to use the indoxyl acetate stain for the demonstration of esterases.

Method 4 - the bromo-indoxyl acetate method (page 206)

The results are expressed as follows:-

pH of the "tris"-HCl buffer	7.0	7.8	8.1	8.5	9.3
Bands appearing	2 bands	2 bands	2 faint bands	2 very faint bands	2 very faint bands

The E_f values of the 2 bands corresponded to those of 2 of the 4 bands that could be detected by Esterase Method 1. These results indicated that a pH of 7 or lower would be the optimum for the enzymes and diazo coupling reactions. It was not possible to make up a

"tris"-HCl buffer of below pH 7 and a "tris"-maleate - NaOH buffer was prepared for a pH range of 5.8 to 8.0. Even after overnight incubation of gel slices in this stain no bands appeared, whatever the pH of the buffer. It was thought that the difference in buffer composition e.g. the presence of maleate, might prevent the bands from developing. Even using phosphate buffer with the same range of pHs, I saw no bands appearing in the gel slices after incubation. After these failures to detect any bands it was decided to repeat the first experiment using the "tris"-HCl buffer system with the pH range between 7.0 and 9.3. Strangely enough no bands were noticed this time even although the electrophoresed cell contents, and the buffered staining solution were apparently identical to those that produced bands originally. In a final attempt to detect bands the concentrations of the NaCl, $CaCl_2$ and the oxidising solution were altered in turn, since it was thought that a slight difference in the concentration of one of these components of the staining solution might be sufficient to cause bands to appear or not, but no bands were detectable. Therefore no explanation could be given as to why the original buffered staining solution demonstrated the presence of bands in the first experiment and could continue to do so on other gel slices until the solution had been used up, and a freshly prepared, apparently identical buffered staining solution could not.

It was concluded that Esterase Method 4, even when it worked, had

no advantage over Esterase Method 1 for the demonstration of esterase bands and no further attempt was made to use the bromo-indoxyl acetate method for the classification of strains of micrococci and staphylococci.

ACID PHOSPHATASE ISOZYMES

Method 1 - the metal salt method (page 206)

When gel slices containing electrophoresed fractions of cell contents of 8 randomly selected strains were incubated in this stain, a high running band was noticed in 2 of the strains. Even in a 15% acrylamide gel, the bands of the 2 strains were still very close to the brown line boundary of the discontinuous buffer system of the gel, and thus it was impossible to know whether the high running components were the same or not. The background stain of the gel slices was very great and could have obscured weak bands, and for this reason this stain was not considered suitable for the detection of phosphatase bands.

Method 2 - the α-naphthyl phosphate method (page 207)

No bands were detected by this method even though the pH was altered and the diazo salt substituted.

Method 3 - the naphthol AS phosphate method (page 208)

The results are expressed as follows:-

pH of "tris"-maleate buffer	5.6	5.7	6.4	6.9	7.4
Bands appearing	no bands	no bands	1 weak band	1 band	1 weak band

The band, which was high running and in a similar position to those demonstrated by Phosphatase Method 1, was detected by this staining method at pHs 6.4, 6.9 and 7.4, but only at pH 6.9 was the band strong enough to be seen clearly. If a gel, however, was prepared from "Cyanogum" dissolved in double strength "tris" - citrate buffer, more phosphatase bands were apparent after staining than if the gel had been prepared from the normal single strength buffer. Also, if a gel, prepared from single strength buffer was electrophoresed, sliced and the slices soaked in a mixture of "tris" - citrate single strength buffer, and the phosphate staining solution, once again, more phosphatase bands appeared. Obviously the "tris" - citrate was required in a higher concentration to stimulate the appearance of more bands, but the significance of the 2 buffer components was not investigated further.

The E_f values of the bands of the phosphatase pattern seemed to be identical, although generally weaker, to those of the esterase bands; this was confirmed when a gel containing the electrophoresed fractions of the cell contents of 8 strains was sliced, and one of the middle slices stained by Phosphatase Method 3, and the other middle slice stained by Esterase Method 1, and the two resultant patterns of isozymes compared and found to be identical.

Although Phosphatase Method 3 was not used for classification of all the strains, it was useful for demonstrating that at least some bacterial enzymes might be able to hydrolyse both esters and phosphates.

β-GLUCURONIDASE ISOZYMES

The post-coupling method (page 208)

With both Fast Blue B and Fast Blue RR salts, the post-coupling method did produce bands on gel slices. Although bands tended to be diffused and weak, they were sufficiently clear to show that β-glucuronidase patterns were the same as esterase patterns for the 15 randomly selected strains examined, when the 2 middle slices of gels were compared. As these β-glucuronidase bands were less distinct than the esterase bands the post-coupling method was not used for classifying the micrococcal and staphylococcal strains.

PROTEOLYTIC ISOZYMES

Method 1: the aminopeptidase, azo-dye method (page 209)

Some bands appeared with E_f values identical to those of bands stained by Esterase Method 1, after incubation in the staining solution, containing buffer at all 3 pHs, but bands were strongest at pHs 7.1 and 7.6. Again in a similar way to Phosphatase Method 3 and the β-glucuronidase method, Proteolytic Enzyme Method 1 was of little use in classification, but the results showed that phosphatase, β-glucuronidase, esterase and proteolytic enzymes have identical E_f values, and it would appear that there are micrococcal and staphylococcal enzymes that are active on a wide range of substrates with ester linkages such as organic esters, phosphates, glucuronides and proteins.

Method 2: the milk overlay method; Method 3: the milk saturation method; Method 4: the gelatin overlay method; Method 5: the gelatin saturation method (page 209)

No bands of clearing were ever seen in milk or gelatin in the use of these methods, despite the fact that gel slices which contained electrophoresed cell contents of strains that visibly broke down milk and gelatin in agar plates (page 143), were tested.

UREASE ISOZYMES

The urea overlay methods (page 211)

Urease activity of micrococci and staphylococci in the urea agar slope medium (page 112) does not correlate well with any other biochemical activity (page 144). The fact that a urease positive strain can be identical with a urease negative strain in other physiological characters may indicate that some urease negative strains may simply lack a urea permease enzyme, and not a urease. It was thought that the detection of urease isozymes in gel slices would show whether a strain possessed a urease or not, since no permease would be required to initiate the break-down of urea to ammonia in gel slices.

The mechanism of this overlay method is that the electrophoresed urease isozymes can act on the urea in a basal medium and release ammonia in discrete zones, shown by a colour change in the pH indicator, at the sites of activity.

All the 5 Urea Basal Media (page 211) could be used to demonstrate

the presence of bands in gel slices, but for the following reasons Basal Medium 5 was chosen as the basal medium for the urea overlay method:-

a) the basal medium required to be at a pH of below 7 (between 5 and 6), since the "tris"-citrate-borate buffered gel slices will automatically turn the whole of the overlay medium weakly alkaline, and thus prevent the detection of all but strong zones of ammonia production.

b) although thymol blue indicator has a more alkaline pH range (8.0 - 9.6) than phenol red (6.8 - 8.4), sufficient ammonia was produced at the sites of activity to create alkaline zones of above pH 9; thymol blue also gives a greater contrast in colour in the gel slices when the pH rises from 7.5 to 9.5.

c) the presence of Ringer's salts solution in the overlay medium had no effect on the production of urease isozyme bands.

The electrophoresed cell contents of 85 randomly selected strains were examined for urease bands in gel slices; 42 strains had the ability to produce low running bands of various E_f values, and 43 had not. Although urease isozyme bands were readily recognisable, they tended to be diffuse, and they faded from the gel slices and the overlay gels within 6 hours. The presence of urease isozyme bands does not correlate well with the positive reactions on urea agar slopes, as shown in Table 39.

Table 39

Comparison of the number of positives occurring in urea agar slopes and the detection of urease isozyme bands in gel slices

85 strains compared							
Urea agar slopes				Urea gel slices			
No. of positives 67		No. of negatives 18		No. of positives 42		No. of negatives 43	
Urea gel positive	Urea gel negative	Urea gel positive	Urea gel negative	Urea slope positive	Urea slope negative	Urea slope positive	Urea slope negative
40	27	2	16	40	2	27	16

It was noticed that of the 67 that were urea slope positive, only 40 strains produced urease bands in gel slices. These results could indicate a) that the urea overlay detection method is less sensitive in detecting urease activity than allowing strains to grow in the presence of urea on slopes, b) that ureases in some strains are present in the soluble cell contents, and in others, ureases are fixed to the insoluble parts of the cells, e.g. the cell walls or cell membranes; the fixed ureases would not be able to be electrophoresed, and therefore no isozyme bands would be visible or c) the ureases of some strains are inducible and would only be detected in gel slices if the cell contents were prepared from cells grown in the presence of urea.

It was also noticed that of the 42 strains that produced urease bands in gel slices, 2 strains failed to produce a positive reaction on the urea slopes. The results of these 2 strains are sufficient to show that a) some strains may lack a urea permease enzyme which would prevent a positive reaction taking place on the urea slopes since the urea would not be able to penetrate the cells, but not in the gels where the urea and the urease enzymes would rapidly come in contact, or b) some cells growing on an unbuffered peptone urea agar initiate complex enzymic biochemical reactions which may mask the effects of the ureases during the incubation period, perhaps by the neutralisation of the ammonia released by an acid, whereas in the urea overlay method, urea is the only substrate present and no other compounds but the break-down products of urea could be released in any quantity.

To try and clarify the situation a selection of 8 strains which showed the anomalous reactions in urea slopes, and urea overlaid gel slices, were examined by the urea tube method (page 212). The results of the detection of urease activity by the 3 methods for these 8 strains is shown in Table 40 .

Table 40

Comparison of results obtained from 3 different methods for detecting urease activity

Arbitrary grouping of strains	Strain no.	Urea agar slope method	Urea tube method	Urea overlay method (gel slices)
Coagulase + ve staphylococcus	328	+	-	-
Coagulase - ve staphylococci	246	+	-	-
	251	+	+	-
	179	+	+	+
Glucose oxidising micrococcus	157	+	+	+
Micrococci unable to oxidise glucose: yellow pigmented	301	+	+	-
red pigmented	332	-	-	+
white pigmented	390	-	-	+

+ = production of ammonia from urea   - = no production of ammonia from urea.

The results of strain nos. 251 and 301 could be explained by the lack of sensitivity of the urea overlay method, or the fixation of the ureases to insoluble parts of the cells, of strain nos. 246 and 328 by the requirement of urea for the induction of the enzyme, and of strain nos. 332 and 390 by the lack of a permease enzyme. Strain nos. 157 and 179 were positive with all 3 methods.

Of these 4 explanations, it was only possible to test whether the ureases in strain nos. 246 and 328 were inducible or not. To this end, these strains were examined by the urease induction method (page 212). If the ureases were induced by the urea, the suspensions in the tubes should have broken down the urea to ammonia; in fact, no break-down was indicated by a change in pH, and it was concluded that the urease enzymes were not inducible.

The urease isozyme bands are unfortunately low running and it was often difficult to decide whether bands from strains had the same E_f value or different ones, and for this reason the urea isozyme results were excluded from the electrophoretic classification scheme. Nevertheless, several points were made with reference to the taxonomic significance of urease isozyme bands.

1) Of the 5 coagulase positive staphylococci examined, no bands were detected, although 4 of these strains were urease positive in urea agar slopes.

2) Of the 13 strains possessing esterase band no. 2*, blood band nos. 1 or 1 and 5, and able to ferment glucose and hydrolyse urea in urea agar slopes, but not able to oxidise mannitol, 11 were able to produce urease bands which seemed to have the same E_f value for each strain.

3) Of the 6 strains possessing esterase band nos. 5 and 9, blood band nos. 3 and 4, and able to ferment glucose and hydrolyse urea in urea agar slopes, but unable to oxidise mannitol, all 6 were able

*the main esterase, and starch hydrolysing isozyme bands and blood bands are shown in Diagram V .

to produce two urease bands, which seemed to have the same E_f values for each strain. The E_f values were different from those of other strains.

4) Of the 9 strains possessing between 3 and 6 of the 12 esterase bands nos. 20 to 31, blood band nos. 3 and 4, and able to ferment glucose and hydrolyse urea in urea agar slopes, each strain produced a urease band, which seemed to have the same E_f value. This E_f value was different from those of other strains.

5) Of the 6 <u>Micrococcus luteus</u> strains tested, 5 were positive in urea agar slopes but only one of these was urease positive in gel slices. This one strain, however, produced 3 low running urease isozymes, the largest number of urease isozymes detected in all the 85 strains examined.

6) Of the 5 <u>Micrococcus roseus</u> strains tested, only 2 were positive in urea agar slopes. One strain produced a strong urease band in gel slices, although it did not hydrolyse urea in urea agar slopes.

7) Other micrococci produced urease isozyme bands of different E_f values, but the results could not be related to any other character.

<u>LIPOLYTIC ISOZYMES</u>

Since many strains of micrococci and staphylococci hydrolyse tributyrin and also possess a lipase enzyme capable of causing opacity in egg yolk when growing on the appropriate media, I considered that it should be possible to demonstrate lipase isozymes in gel slices.

Method 1: the 2-naphthyl laurate method; Method 3: the Nile Blue sulphate tributyrin overlay method; Method 4: the tributyrin saturation method (page 212)

No bands were detected by any of these methods.

Method 2: the tributyrin overlay method (page 212)

No bands were detected by Method 2a but in Method 2b a band of clearing in 2 out of 22 strains was noticed in the opaque Tributyrin Agar that had penetrated the gel slices. Clearly 56°C for 10 minutes did not denature the tributyrinase isozymes, and apparently allowed penetration of the molten tributyrin into the gel slices so that even a low concentration of enzyme could produce some clearing, since it did not need to diffuse to the surface of the gel in order to come in contact with the substrate. The tributyrinase isozymes had the same R_f value. One of the strains was a coagulase positive staphylococcus, which hydrolysed tributyrin in the plate (page 112) and caused opacity in egg yolk agar (page 112), and the other strain was a coagulase negative staphylococcus which hydrolysed tributyrin in the plate but failed to cause opacity in egg yolk agar. Of the other 20 strains, which did not produce any lipase isozyme bands, 18 hydrolysed tributyrin in the plate. It appears, therefore, that the presence of tributyrin isozymes does not correlate well with positive results of strains growing in Tributyrin Agar and egg yolk agar. It may well be that the tributyrin overlay method is not the best method for detecting

lipolytic isozymes, and it did not seem a very suitable method for taxonomic experiments with micrococci and staphylococci; the method was not used further.

SUCCINIC DEHYDROGENASE ISOZYMES

Succinic dehydrogenase is the one histochemically demonstrable dehydrogenase system that requires no co-enzymes, at least in human tissue sections, and it seemed to be a suitable one to detect in gel slices.

Method 1: the Nitro BT method and Method 2: the modified Nitro BT method (page 214)

When gel slices were incubated in phosphate buffered staining solution (Succinic Dehydrogenase Method 1) at pHs 6.7, 7.0, and 7.7, high running opaque bands were detected, but this did not occur in "tris"- HCl buffered staining solution (Succinic Dehydrogenase Method 2) at the same pHs. Two high running opaque bands could be demonstrated for all the 20 randomly selected strains of micrococci and staphylococci examined by the first method, but it was impossible to differentiate between strains by this method. Blue formazan bands, however, should appear at sites of succinic dehydrogenase activity and not these opaque bands. To show whether these opaque bands, produced by Succinic Dehydrogenase Method 1, were enzymic in origin or not the effect of heat and chemical inhibitors on the formation of the bands was examined. The Succinic Dehydrogenase Heating Experiment (page 221) was carried out. Even

after subjection to 109 °C for 1 hour the electrophoresed cell contents still retained the ability to produce opaque bands. In plant and animal cells malonate is known to competitively inhibit succinic dehydrogenase (Baldwin, 1960; page 33), and it was found pre-incubation of gel slices in 10^{-2}M malonate for 1 hour at 37°C followed by incubation in a mixture of staining solution and 10^{-2}M malonate resulted in complete inhibition of the opaque bands. Inhibition did not take place at lower concentrations of malonate, or, if there was no pre-incubation of the gel slices, even at 10^{-1}M concentration. If gel slices were incubated in the same way in 10^{-2}M potassium cyanide and 3M urea, complete inhibition was again achieved. Although malonate is a specific inhibitor for succinic dehydrogenase the cyanide and the urea are not known to inhibit the dehydrogenase, whereas they are known to inhibit other enzyme systems. In view of the very high heat resistance of the agent that produces the opaque bands and also the nature of the inhibition, I think that the reaction is likely to be non-enzymic. It is possible that the high running components precipitate out chemically in the presence of the staining solution, and the pre-incubation of gel slices in the inhibitors merely results in the high running components being in a chemical reaction in such a way that they no longer precipitate out in the staining solution.

Therefore, although Succinic Dehydrogenase Method 1 produces

bands these were not considered to have originated from a succinic dehydrogenase enzyme and it was decided not to use this method for classification purposes.

LACTIC DEHYDROGENASE ISOZYMES

In contrast to succinic dehydrogenase both lactic dehydrogenase and malic dehydrogenase require nicotinamide adenine dinucleotide (NAD) as a coenzyme and phenazine methosulphate (PMS) as a part of the stain; the dehydrogenases reduce PMS to a dye which couples to a tetrazolium salt and allows the sites of dehydrogenase activity to be seen.

The Nitro BT method (page 214)

Gel slices containing electrophoresed cell contents showed blue formazan bands for some strains, but not for others; of the 48 randomly selected strains of micrococci and staphylococci, only 18 produced bands. Four coagulase negative staphylococci (strain nos. 12, 13, 54 and 72) which are physiologically related, and have 3 esterase bands in common, produced the same lactic dehydrogenase pattern (Pattern 1, Diagram VI).

Coagulase positive strain nos. 44, 51, 58, 88 and 106 produced lactic dehydrogenase Pattern 2(Diagram VI), and 2 other coagulase positive strains (nos. 62 and 100) produced the related lactic dehydrogenase Pattern 3 (Diagram VI). Strain no. 152 (coagulase positive) was the only strain to show a weak pattern, that of Pattern 2. One other coagulase positive strain (no. 101)

produced a different but related pattern (Pattern 4, Diagram VI).

Thus as far as coagulase positive strains are concerned, of the 10 strains examined, 1 strain (no. 49) produced no lactic dehydrogenase pattern at all, 6 strains showed Pattern 2, 2 strains showed Pattern 3, and 1 strain Pattern 4. It is interesting to note that a coagulase negative strain (no. 110), which oxidised mannitol gave the same pattern (Pattern 4) as coagulase positive strain no. 101; they also have an esterase band, and a catalase band in common.

Strain nos. 91 and 105, which have little in common physiologically, produced the same high running lactic dehydrogenase band (Pattern 5, Diagram VI).

A low running band (Pattern 6, Diagram VI) was produced by the biochemically weak strains, nos. 147 and 150, which also have 2 esterase bands in common.

The first 4 of these patterns seem to be related, and the 13 strains producing Patterns 1 - 4 tended to be biochemically active e.g. 9/13 strains fermented mannitol, 11/13 strains oxidised mannitol, 12/13 strains fermented glucose, and 9/13 strains produced coagulase.

Although this stain was not used to classify all 406 strains it has been shown that isozymes of lactic dehydrogenase are of significance in the taxonomy of micrococci and staphylococci.

MALIC DEHYDROGENASE ISOZYMES

The Nitro BT method (page 215)

For the 8 randomly selected strains of micrococci and staphylococci examined the malic dehydrogenase patterns on gel slices, containing electrophoresed cell contents, were identical to those obtained by the lactic dehydrogenase stain. It seems that dehydrogenase isozymes of micrococci and staphylococci have the ability to dehydrogenate lactate and malate. Since the malic dehydrogenase stain has no advantage over the lactic dehydrogenase stain, however, for classification, no further experimental work was done with this stain on enzyme systems in gel slices.

PEROXIDASE ISOZYMES

Method 1: the "nadi" method (page 215)

In 1885, Ehrlich discovered the indophenol reaction by noting that a blue dye was formed upon the injection of α-naphthol and NN'dimethyl-p-phenylenediamine into living tissue. The formation of the dye, indophenol blue, has been referred to as the "nadi" reaction from the first two letters of "naphthol" and "diamine". Although the reaction was originally thought to be specific for cytochrome oxidase (Keilin & Hartree, 1938), the "nadi" reaction has been used for the demonstration of other oxidase systems, as peroxidase (Lillie, 1954). I found, however, that no bands appeared in gel slices when Lillie's (1954) stain was applied, and I had to modify the stain (page 216) to allow bands to be detected.

Twenty four randomly selected strains of micrococci and staphylococci, when tested by this modification, yielded not only high running bands that corresponded to those that were produced by Peroxidase Method 2 (page 216) Cytochrome Oxidase Methods 1 and 2 (page 217), and Blood Band Detection Method (page 219), but also relatively low running bands which had identical E_f values to those that could be demonstrated by the Catalase Detection Method (page 216) and Cytochrome Oxidase Methods 1 and 2. These low running bands are equivalent to the strongest of the bands produced by the Blood Band Detection Method. The high running bands were of little use in classification since all the strains examined produced 2 bands, and these had the same E_f values for each strain. The low running bands could have been used in the electrophoretic classification scheme of the 406 strains (page 277), but the Blood Band Detection Method detected patterns, clearer than those detected by the "nadi" method, and these patterns were used in classification instead.

Method 2: the o-dianisidine method (page 216)

In all the 24 randomly selected strains examined by this method in gel slices 2 brown high running bands were detected for every strain and, as mentioned above, these bands were of little use in classification.

CATALASE ISOZYMES

Catalase is an important enzyme in some groups of bacteria, including the *Micrococcus-Staphylococcus* group, and isozymes should be

detectable in gel slices, especially as catalase isozymes of other bacteria have been demonstrated in starch gel slices (Table 38).

The H_2O_2 method (page 216)

This method has not been used to demonstrate catalase bands in acrylamide gel slices before. In fact, although catalase bands were seen in both starch and acrylamide gel slices containing electrophoresed cell contents of micrococci and staphylococci and faded equally quickly from slices of both support media, the bands appearing in the acrylamide gel slices were less diffuse than those occurring in starch gel slices. Therefore, I chose to detect catalase isozyme bands by the H_2O_2 method in acrylamide gels, rather than in starch gels. Catalase bands, detected in acrylamide gel slices, can be seen in Plate 5 .

Catalase bands correspond to the low running peroxidase bands, cytochrome oxidase bands, and the strongest of the low running blood bands (page 247).

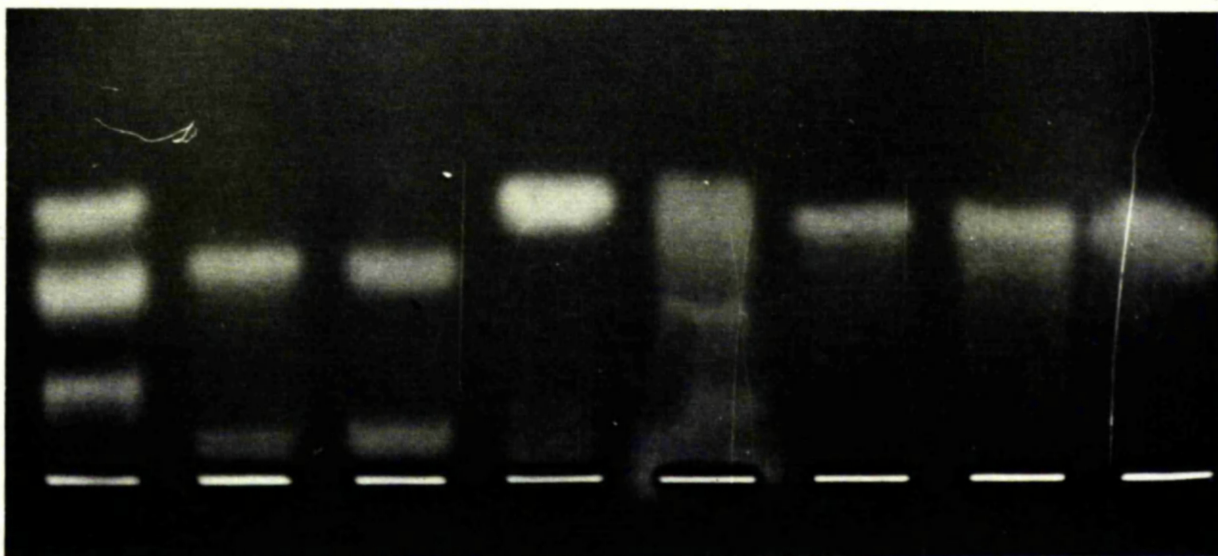
For reasons given on page 258 catalase isozyme bands were not used as characters in the electrophoretic classification scheme (page 177). Catalase bands, detected in acrylamide gel slices, can be seen in Plate 5 .

Comparison of the physiological and electrophoretic methods of detecting catalase

Attempts were made to demonstrate catalase bands in the electrophoresed

Plate 5

Acrylamide gel slice showing catalase isozyme bands



Electrophoresed cell contents of 8 randomly selected strains of micrococci and staphylococci

cell contents of 110 strains - 104 of these strains produced one or more catalase bands in gel slices, 6 did not. The results of the physiological and electrophoretic detection of catalase in these strains is shown in Table 41.

Table 41

Comparison of strains producing catalase enzyme from growing cells and catalase isozyme bands from electrophoresed cell contents

	Catalase enzyme production + ve	Catalase enzyme production - ve
Catalase isozyme bands + ve	103 strains	1 strain
Catalase isozyme bands - ve	4 strains	2 strains
Total no. of strains compared = 110		

There is a close but not complete correlation (95.5%) between strains producing catalase enzyme from growing cells and catalase isozyme bands from electrophoresed cell contents. One of the strains unable to produce catalase from colonies growing on agar, no. 6, was physiologically similar to the other catalase negative strains, nos. 31 and 89 (page 159). Yet strain no. 6 produced a catalase band typical of many coagulase negative staphylococci, whereas strain nos. 31 and 89 did not produce any visible catalase bands, as would be expected. From this experiment, therefore, catalase production, either as effervescence of oxygen from hydrogen peroxide by colonies growing on agar, or as isozyme bands appearing in gel slices containing electrophoresed cell contents, should be used with caution

as a main character in a classification scheme (page 158).

CYTOCHROME OXIDASE ISOZYMES

Cytochrome oxidase is an enzyme that occurs in the terminal oxidation pathway of aerobic respiration of cells of plants, animals, aerobic and facultatively anaerobic bacteria; consequently it should be present in electrophoretically fractionated cell contents of micrococci and staphylococci.

Method 1: the p-aminodiphenylamine method and Method 2: the tetrazolium method (page 217)

Both of these methods gave results, but the latter method demonstrated bands of greater definition than the former. The patterns produced in gel slices were equivalent, in all 8 randomly selected strains of micrococci and staphylococci examined, to those produced by Peroxidase Method 1 (page 215), and since there was no advantage over this peroxide method, Cytochrome Oxidase Methods 1 and 2 were not used in classification.

MONAMINE OXIDASE ISOZYMES

Monamine oxidase is an important mammalian enzyme which converts amines to aldehydes and is likely to be present in the cell contents of micrococci and staphylococci.

The tetrazolium method (page 218)

On staining gel slices containing the electrophoresed cell contents of 16 randomly selected strains, with Monamine Oxidase Stain, no blue bands, but high running opaque bands, equivalent to those

detected by Succinic Dehydrogenase Method 1 (page 214) were seen.

According to Burstone (1960, page 437), 10^{-2}M p-chloromercuribenzoate and 3M urea are specific inhibitors for mammalian monamine oxidase. In gel slices the opaque bands were completely inhibited by 10^{-1}M p-chloromercuribenzoate and 3M urea and partially inhibited by 10^{-2}M p-chloromercuribenzoate. These inhibition experiments would indicate that the opaque bands were caused by enzymic activity, but heating experiments showed otherwise. Like the opaque bands produced by Succinic Dehydrogenase Method 1 these bands are formed even after the cell contents have been autoclaved at 109°C for 30 minutes (page 243). It is difficult to conceive of an enzyme capable of resisting this temperature, and the reaction could be explained by the reason given on page 243. The Monamine Oxidase Method was not used further for staining gel slices.

DOPA OXIDASE ISOZYMES

This enzyme is commonly found in mammalian tissues and is capable of breaking down dihydroxyphenylalanine to a dark pigment, melanin. Since many micrococci and staphylococci are pigmented it is possible that these organisms produce melanin or related compounds as pigments and would possess DOPA oxidase enzyme.

THE DOPA method (page 218)

The cell contents of 16 randomly selected strains, some pigmented, some not, producing various colours of pigment, were electrophoresed on acrylamide gels and the slices stained by the DOPA method. No

bands were seen in the gel slices and the stain was not used any further.

STARCH HYDROLYSING ISOZYMES

The break-down of starch in the agar plate (page 106) seems to be one of the few demonstrable biochemical reactions that strains of *Micrococcus lutaus* and *Micrococcus roseus* are capable of doing in routine physiological tests (page 136). It was thought that classification of these biochemically weak strains could be aided by examining gel slices for the presence of starch hydrolysing isozymes.

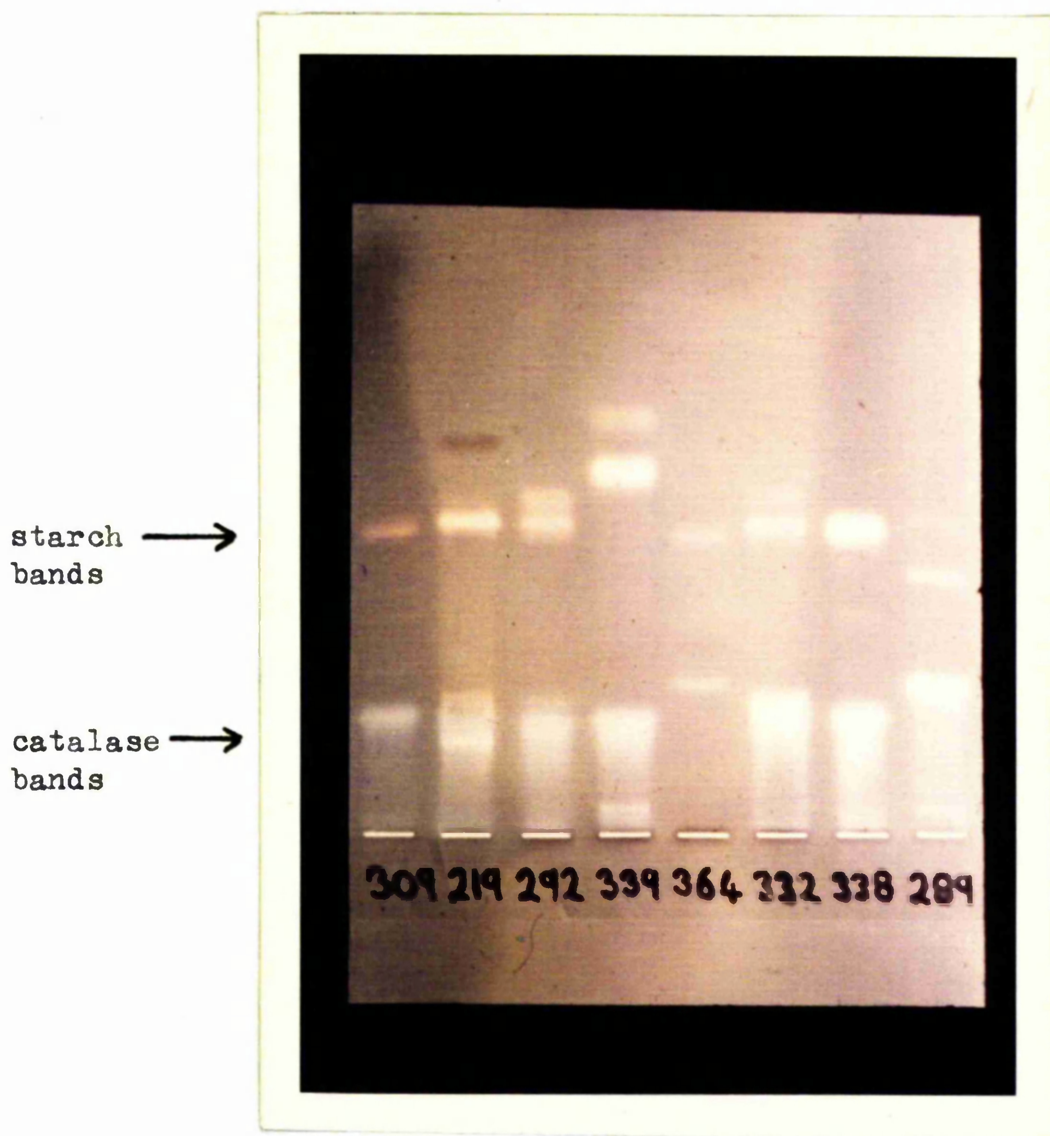
The H_2O_2 method (page 218)

Yellow, orange, red and colourless bands, different from catalase bands because they do not fade quickly, were detected in acrylamide gel slices (Plate 6), but not in starch gel slices, by the H_2O_2 method for a limited number of strains.

In order to see whether these bands were caused by the break-down of starch, as I suspected, or not, first of all a comparison was made of the strains that produced bands in gel slices, and those that hydrolysed starch in the agar plate. All the strains which hydrolysed starch in agar, the 4 strains of *Micrococcus luteus* which did not hydrolyse starch in agar, and randomly selected strains of micrococci and staphylococci which did not hydrolyse starch in agar (a total of 144/406 strains) were examined for starch bands in acrylamide gel slices.

Plate 6

Acrylamide gel slice stained by the H_2O_2 method showing starch hydrolysing isozyme bands



The numbers refer to strain numbers. Strain no. 289 is a yellow pigmented, glucose oxidising micrococcus. All the other strains are micrococci unable to utilise glucose:-

strain nos. 309, 338 and 364 are yellow pigmented;

strain nos. 219, 292 and 332 are red pigmented *Micrococcus roseus* strains;

strain no. 339 is a red pigmented *Micrococcus radiodurans* strain.

Starch hydrolysing isozyme bands are red, orange or yellow coloured, or clear, and do not fade quickly. The clear low running bands are catalase bands; these fade within a few minutes (cf. Plate 7).

Of the 144 strains examined,

33 strains hydrolysing starch in agar, also produced starch bands,

7 strains hydrolysing starch in agar, failed to produce any starch bands,

5 strains failing to hydrolyse starch in agar, produced starch bands, and

99 strains failed both to hydrolyse starch in agar, and produce starch bands.

A more detailed comparison is shown in Table 42.

Table 42

Comparison of numbers of strains producing starch bands in gel slices and strains hydrolysing starch in agar

Arbitrary grouping of strains	No. of strains examined compared	Hydrolysis of starch in agar plates		Production of starch bands in gel slices	
		+	-	+	-
coagulase + ve staphylococci	4/21	1	3	0	4
coagulase - ve staphylococci	58/207	9	49	1	57
glucose oxidising micrococci	38/130	3	35	1	37
micrococci unable to oxidise glucose: red pigmented	10/10	10	0	9	1
yellow pigmented	26/26	22	4	24	2
white pigmented	5/9	0	5	0	5
other pigments produced	3/3	1	2	2	1

+ = positive reaction - = negative reaction

These results show that there is a close but not complete correlation, between the number of strains hydrolysing starch in agar plates and the number of strains producing starch bands. It is in the red and yellow pigmented micrococci unable to utilise glucose that the correlation is closest. The results of the anomalous starch results could not be accounted for, but the reasons put forward to explain the anomalous urease results (page 238) could equally well be applied to these results.

The next step was to see if the patterns detected by the Starch Hydrolysing Enzyme Detection Method were caused by enzymes. Unfortunately there is no known specific inhibitor for starch hydrolysing enzymes (amylases), but mercuric chloride, which is a general inhibitor of many enzyme systems, was used. Pre-incubation of gel slices in 0.002M mercuric chloride for 30 minutes, followed by soaking of the slices in dilute boiled starch, containing the same concentration of inhibitor, overnight inhibited the production of any starch bands, whereas lower concentrations of mercuric chloride did not prevent the appearance of the bands at all. Starch bands were also prevented from forming if the cell contents were heated for 1 hour at 60°C and weak bands appeared after cell contents were heated for only 5 minutes at 60°C. In addition it was found that a comparison of the positions of bands as detected by Starch Hydrolysing Enzyme Detection Method and those detected by Protein Stain indicated that the starch bands are caused by agents protein in nature.

These experiments indicate that the agents responsible for these bands appearing are starch hydrolysing isozymes, which are inhibited by mercuric chloride and are heat sensitive. In my opinion, therefore, the mechanism of the formation of bands by the Starch Hydrolysing Enzyme Detection Method is as follows:-

the isozymes cause the break-down of starch in the acrylamide gel slices during the incubation period, to give first of all dextrins, and then oligosaccharides, maltose or glucose. When the iodine is released by the peroxide from potassium iodide, it reacts with the starch in the gel slices to give a dark blue colour, but in contrast, where the starch has been hydrolysed to dextrins, red, orange and yellow bands appear; complete hydrolysis of the starch to oligo- or disaccharides results in the colourless bands. Whether one or more amylase enzymes is active in the isozymes it is not possible to say, but since some bands appear to be dark red and others colourless (Plate 6) there is an indication that there is more than one type of amylase in some of the isozymes.

Starch hydrolysing isozyme bands were used in the electrophoretic classification scheme of micrococci and staphylococci (page 277), and were of particular use in classifying strains of Micrococcus luteus and Micrococcus roseus. These bands can be seen in Plate 6 , and in Diagram V .

There is one final point of interest concerning the H_2O_2 method of detecting starch hydrolysing enzyme bands. If the iodine stained

gel slices, which had demonstrated starch hydrolysing enzyme bands (Plate 6), were soaked in water for up to a week, the background stain gradually disappeared but numerous dark blue bands remained (Plate 7). Since these bands formed patterns similar to those stained by Protein Stain, the following experiment was carried out:-

The cell contents of 8 strains (some producing starch hydrolysing isozymes, some not) were electrophoresed on an acrylamide gel and the gel sliced. One of the middle slices was stained by the H_2O_2 method and the slice decolourised in water until these dark blue bands appeared, and the other middle slice was stained by Protein Stain. By comparing the 2 stained slices, the patterns were considered to be identical, and it was concluded that dark blue bands appearing during staining by the H_2O_2 method were composed of protein, and that the blue black starch-iodine complex absorbed on to protein bands. These dark blue bands stained by the H_2O_2 method are not permanent, however, as they fade after a day or two longer in water, but they reappear in the presence of dilute iodine.

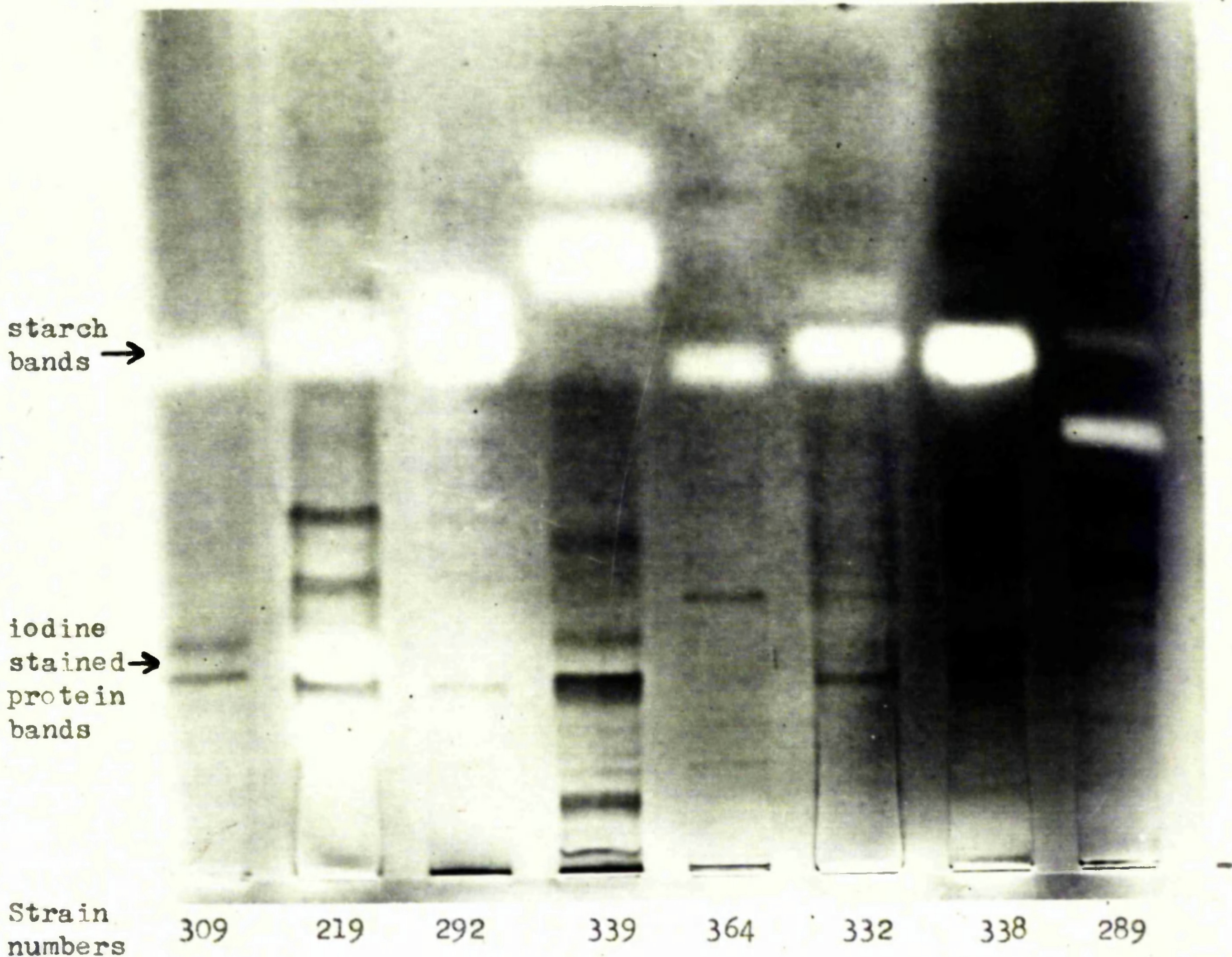
The H_2O_2 method is unique in that it can detect more than one recognised enzyme system, and can be used to demonstrate the presence of protein bands, catalase bands and starch hydrolysing enzyme bands.

<u>BLOOD BAND ISOZYMES</u> (page 219)

This method was evolved out of an attempt to demonstrate haemolysins in gel slices. It is known that staphylococcal haemolysins are active only on certain blood species (page 57), and it was thought

Plate 7

Acrylamide gel slice stained by the H_2O_2 method showing starch hydrolysing isozyme bands (clear zones) and iodine stained protein bands (dark zones)



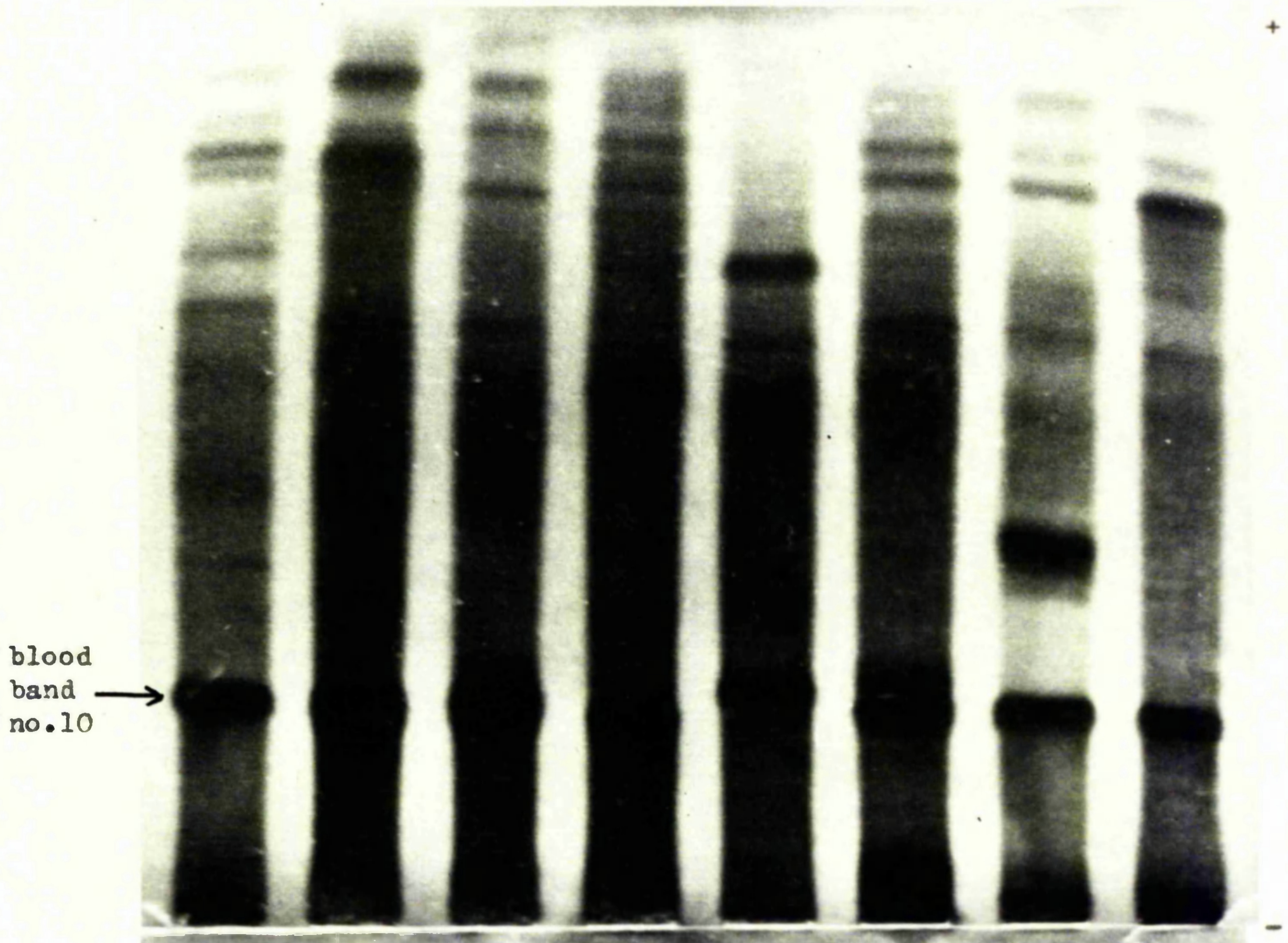
Electrophoresed cell contents of the same strains as in Plate 6. It is worth noting that the catalase bands shown in Plate 6 faded before this photograph was taken.

that if gel slices, containing electrophoresed cell contents, were overlaid with agar, containing different species of blood, areas of clearing might occur in some of the blood agar overlays and not in others. Horse, human, rabbit and sheep blood agar sheets were overlaid with strips of a gel slice, each strip containing the same electrophoretic fraction of the cell contents of one haemolytic Staphylococcus aureus strain, and incubated. In fact, no haemolytic zones appeared in any of blood agar overlays, but the gel strips had become opaque all over, except for bands of clearing - now called "blood bands" - as described in the Materials and Methods section page 219, and shown on Plate 8. The transparent bands formed identical patterns with all the species of blood used in the overlays. Thus, although haemolysins active in different species of blood could not be identified, I thought that the bands could be of taxonomic significance, and when I repeated the experiment, using the electrophoresed cell contents of 8 randomly selected strains in acrylamide gel slices overlaid with agar containing one species of blood (horse), I found that different blood band patterns developed for each strain, with some strains having 1 or 2 bands in common.

I considered from the experiment, that blood bands could be useful characters in a classification scheme, and accordingly I examined all 406 strains for blood band patterns. Many of the strains produced between 5 and 7 different blood bands. The R_f values of the strongest low running blood bands were identical to those of catalase

Plate 8

Acrylamide gel slice showing blood band isozymes



Electrophoresed cell contents of 8 *Micrococcus luteus* strains. These strains produce different but related patterns of blood bands.

The strong low running blood bands (usually blood band no. 10 in strains of *Micrococcus luteus* and *M. roseus*) have the same E_f values as the catalase bands produced by the same strains.

The photograph was taken with transmitted light against a dark background.

bands in 100 strains compared, and the heat resistances and degrees of chemical inhibition are the same for agents causing catalase bands and blood bands in gel slices (page 268). It appears, therefore, that catalase bands are equivalent to the strongest low running blood bands. Since catalase bands fade quickly after their formation in acrylamide and starch gels, and more blood bands were detected per strain than catalase bands, I chose blood bands in preference to catalase bands as taxonomic characters for classifying all 406 strains in my electrophoretic classification scheme (page 277), as there was no point in testing strains for both types of band.

I considered that the comparison of E_f values of all the blood bands of all the 406 strains for classification purposes was impossible; I used the E_f values of the strongest blood bands, usually 1 or 2, for the strains that could be classified on many positive physiological characters, e.g. strains of *Staphylococcus aureus* and *S. epidermidis*; for the relatively few strains that exhibited a small number of physiological characters e.g. strains of *Micrococcus luteus* and *M. roseus*, I compared the E_f values of all the blood bands (between 3 and 7 blood bands per strain). Although only a few strains were classified with bands additional to the strongest blood bands, the band numbers assigned to them were valid, since no other micrococcal or staphylococcal strain produced any blood bands of the same E_f values.

Blood bands can be seen on Plates 8 and 9 . Diagram V shows patterns of blood bands, typical of certain groups of strains; blood band numbers are also shown.

Mechanism of the formation of blood bands

At this stage it seemed that the formation of blood bands was caused by more than simply just the effect of lysis of red cells and probably depended on an alteration in the properties of the haemoglobin released as well. The red colour, which had penetrated the acrylamide gel after overnight incubation at 37°C, could have come only from ruptured red blood corpuscles, which owing to their relatively large size could not have escaped from the agar in any quantity. Although it was likely that haemoglobin was responsible for the red colour and opacity in the gel slices, I thought it was important to check the significance of the other components of blood. To this end, the plasma supernatant of spun whole blood (horse) was taken and added to the basal medium; the plamma overlay did not produce any visible effect in acrylamide gel slices after incubation. Just enough distilled water was added to the spun red cells in order to lyse them, and the grey insoluble cell fragments spun down and the red supernatant removed. Both the soluble and the insoluble red cell fractions were mixed separately with the basal medium in the same way as for the whole blood. The insoluble cell fraction overlay agar was faintly opaque before it came in contact with the gel slices but after incubation with the

slices no red colour or bands were seen. The agar with the cell supernatant fraction, containing haemoglobin, had a distinct red colour before layering on to the gel slices, and after incubation the gel slices demonstrated the characteristic opacity with many bands of clearing showing identical patterns to those seen with whole blood. Although it was not possible to examine the effect of a pure preparation of soluble haemoglobin in an agar overlay on gel slices, these experiments have indicated that haemoglobin is probably responsible for the opacity in the gel slices, after incubation at 37°C.

Now it is already known that whereas sheep haemoglobin is very stable, human and horse haemoglobin denature readily and will give an insoluble smoky opacity in agar after incubation at 37°C (Nelson, 1958a, b; Hutchison, 1962). This information explains why horse, human and probably rabbit blood overlays produce a smoky opacity in acrylamide gel slices, and also why sheep blood overlay does so only very weakly, since it is much more resistant to denaturation.

It would seem therefore, that the horse, human and rabbit cells that are in contact with the gel slices lyse, allowing the haemoglobin released to diffuse into the slices. Since lysis of the red cells does not take place without the presence of an electrophoresed acrylamide gel slice (starch gel slices do not produce blood bands) and an incubation period of over 12 hours at 37°C, haemolysis is achieved by the presence of "tris"-citrate-borate

buffer (about pH 8.5), the low concentration of cyanide from the gel monomer or the catalysts in the acrylamide gel, and the 37°C temperature during the overnight incubation. The haemoglobin in the gel slices then undergoes denaturation during the incubation period to form the insoluble opacity, caused, according to Hutchison (1962), by the agglomeration of haemoglobin molecules. The electrophoresed cell contents of all the strains examined contained certain substances, possibly enzymes, which prevented the formation of, or caused the break-down of this denatured haemoglobin.

If the blood band pattern of a middle slice of an acrylamide gel is compared with a protein pattern, stained by Protein Stain, of the other middle slice of the same gel, each of the blood bands has a corresponding protein band of the same E_f values. Therefore, it seems that these blood bands are protein in nature. To try and establish if these bands are enzymic, the effect of heat and enzyme inhibitors on the formation of the bands was examined. As can be seen from Table 45 and Diagram VII, heat prevents the formation of blood bands and each band may differ in its sensitivity to heat between the time-temperature combinations of 1 hour at 60°C to 5 minutes at 80°C. The formation of blood bands was also prevented by 10^{-1}M potassium cyanide, and partially prevented by 10^{-2}M cyanide. These experiments indicate that blood bands are caused by isozymes, and their relationships with catalase bands and high and low running bands detected by Peroxidase Method 1 are discussed later (page 273).

The strongest low running blood bands also correspond to bands detected by Cytochrome Oxidase Method 2. The close relationships between blood bands and these other enzyme systems indicate that blood band isozymes are catalases, oxidases or peroxidases. The mechanism of the prevention of the formation of, or the break-down of the denatured haemoglobin, however, is unlikely to depend on the release of oxygen from H_2O_2 or peroxides, since haemoglobin has a catalase-peroxidase system of its own (the addition of H_2O_2 to crude haemoglobin results in effervescence).

It would seem much more likely that the bands are formed by the action of proteolytic enzymes. To find out if this is so, an overlay agar sheet, containing crude haemoglobin was prepared, and the haemoglobin was allowed to be denatured and become opaque by incubation of the agar sheet at 37°C overnight. Then, the agar sheet was washed thoroughly to remove any undenatured soluble haemoglobin, and the sheet overlaid with an acrylamide slice, containing electrophoresed cell contents. After incubation overnight at 37°C, the gel slice and the agar sheet were both examined for bands of clearing, but none were visible. It seems unlikely, therefore, that blood bands are produced by the action of proteolytic isozymes, although this last experiment may not have been sensitive enough to detect proteolysis of the denatured haemoglobin (compare results with those of Proteolytic Enzyme Detection Methods page 234). Other pieces of experimental evidence

to show that blood bands are not caused by proteolytic isozymes, are that a) all the 406 strains produce blood bands, but only some of the strains are proteolytic as shown by physiological tests (page 43), and b) the patterns of bands produced on gel slices by Proteolytic Method 1 (page 233) correspond to esterase patterns and not blood band patterns.

Unfortunately, there is no positive evidence to show what the mechanism of blood band formation is, and it is possible to speculate only on the negative results. All the information indicates that the agents causing the bands of clearing are enzymes which may be protein-linked with components having catalase-oxidase-peroxidase activity, but not necessarily playing any part in the formation of blood bands. Since it seems that blood band isozymes are not active in breaking down denatured haemoglobin, as the last experiment demonstrated, then they must prevent, in some way, the formation of the insoluble opaque haemoglobin. The mechanism of the prevention of the haemoglobin from denaturing to give clear bands is still obscure.

THE SIGNIFICANCE OF HEAT AND ENZYME INHIBITORS IN THE CLASSIFICATION OF ESTERASES

Heat

Baillie & Norris (1963) examined the effect of heat on esterases of *Bacillus cereus*, and they found that a high running esterase band, associated with vegetative cells, was inactivated by heating at 50°C

for 5 minutes, and a low running esterase band, associated with sporing cells was inactivated at 60°C for 5 minutes. Cann & Willox (1965) also examined the effect of heat on the formation of esterase bands, but this time on Mycobacterium species; they found that most esterase isozymes were resistant to heating at 60°C for 4 hours, and some were even resistant to heating at 100°C for 10 minutes.

To find out whether the heat resistances of micrococcal and staphylococcal esterases were similar in any way to those of Bacillus and Mycobacterium esterases, the Esterase Heating Experiment (page **220**) was carried out. The results are recorded in Table 43 .

Table 43

The heat resistances of micrococcal and staphylococcal esterases

Esterase Pattern	Time (minutes)	30	5	5	60	120	240	5	30	5
	Temperature (°C)	45	50	60	60	60	60	80	80	100
High running bands		+	+	±	∓	∓	∓	∓	-	-
Low running bands		+	+	-	-	-	-	-	-	-

\+ = all the bands present at full strength ± = all bands weak

∓ = all bands very weak - = no bands present at all

All the esterases appeared to be active after heating at 5 minutes for 50°C, but only the high running esterases were resistant to 60°C for 5 minutes and 80°C for 5 minutes, although the bands appeared very faint. It would seem that the low running esterases have a

slightly lower heat resistance than the high running ones. It is difficult to compare these results with those of Baillie & Norris (1963) and Cann & Willox (1965), and the only esterases with comparable heat resistance are the low running esterases of micrococci and staphylococci and the high running ones of the vegetative cells of *Bacillus cereus*.

Chemical enzyme inhibitors

The selective action of inhibitors have not been used, as far as is known, for the classification of bacterial esterases, although they have been used for the identification of different mammalian esterases in tissue sections. Four types of mammalian esterases have been distinguished by inhibitors. Mammalian esterases are primarily differentiated into 2 groups by the action of eserine (Pearse, 1960; page 483) - esterases which are sensitive to 10^{-5}M eserine are considered to be cholinesterases; these are enzymes which hydrolyse acetylcholine and play an important part in the neuromuscular system in animals - esterases which are insensitive to 10^{-5}M eserine are considered to be non-specific esterases active on many ester substrates. The non-specific esterases can be differentiated further into A-, B-, or C-esterases by the use of other inhibitors as shown in Table 44.

Table 44

Classification of mammalian esterases by chemical inhibitors (after Pearse, 1960; page 483)

Enzyme †	Complete inhibition by
A-esterase	10^{-3}M DPF*, 10^{-2}M E 600 10^{-4}M PCMB
B-esterase	10^{-5}M DPF, 10^{-5}M E 600
C-esterase	10^{-3}M DPF, 10^{-2}M E 600 10^{-2}M -phenylpropionic acid (activated by 10^{-4}M PCMB)

* the full names of these inhibitors are given on page 222.

† the substrates used are α-naphthyl acetate, naphthol AS acetates and indoxyl acetates.

The same concentrations of inhibitors that are used for the differentiation of mammalian esterases in tissue sections were used on isozymes of bacterial esterases in gel slices. Gel slices, containing electrophoresed cell contents of 12 strains of micrococci and staphylococci, were pre-incubated in the staining solution for Esterase Method 1; control gel slices were pre-incubated in water before staining.

No micrococcal or staphylococcal esterases were inhibited by 10^{-5}M eserine and, therefore none of the esterases were cholinesterases, which is not surprising since bacteria have

neither nerves nor muscles, and would not require acetylcholine.

Gel slices were pre-incubated in DPF in the following concentrations:- 10^{-3}M, 5×10^{-4}M, 10^{-4}M, and 10^{-5}M. None of the 17 esterase bands detected on the control slice failed to appear after being in contact with 10^{-5}M DPF. With the higher concentrations some of the esterases were inhibited: the bands that were weak in the control slice did not appear after pre-incubation in the higher concentrations of DPF, and only 5 strong high running bands were visible after pre-incubation of gel slices in 10^{-3}M DPF. It seemed, therefore, that the degree of inhibition of these esterases was proportional to the amount of esterase present rather than to the type of esterase present. From these results it was at least possible to say that there were no B-esterases.

To distinguish between possible A- and C-esterases gel slices were first of all pre-incubated in 10^{-5}M DPF for 1 hour and then were incubated in the esterase staining solution, containing either 10^{-4}M PCMB or 10^{-2}M -phenylpropionic acid. The subsequent incubation in 10^{-4}M PCMB prevented the formation of 3 bands and none of the esterase isozymes was activated by this chemical; the presence of -phenylpropionic acid prevented the formation not only of the same 3 bands but also 2 others. From these results it does not seem possible to divide micrococcal and staphylococcal esterases into A-, B- and C-esterases, by inhibitors, since

A-esterases should be inhibited by PCMB, and C-esterases should be inhibited by 3-phenylpropionic acid but activated by PCMB.

HEAT RESISTANCE AND INHIBITION EXPERIMENTS ON PEROXIDASES, CATALASES AND BLOOD BANDS

Heat

In order to find out whether the patterns in gel slices detected by Peroxidase Methods 1 and 2, Catalase Detection Method and Blood Band Detection Method were enzymic in origin or not, the heat resistance of, and the effect of an enzyme inhibitor on, agents which cause these patterns was examined.

First of all, Peroxidase, Catalase and Blood bands Heating Experiment (page 221) was carried out. The results are expressed in Table 45.

Table 45

Heat resistance of agents that produce patterns in gel slices

Pattern / Time (minutes)	30	5	5	60	120	240	5	10	30
Temperature	45°C	50°C	60°C	60°C	60°C	60°C	80°C	100°C	109°C
High running bands (Peroxidase Method 2)	+	+	+	+	+	+	+	+	+
High running bands (Peroxidase Method 1)	+	+	+ -	-	-	-	-	-	-
Low running bands (Peroxidase Method 1)	+	+	+	+ -	+ -	+ -	- +	-	-
Low running bands (Catalase Detection Method)	+	+	+	+ -	+ -	+ -	- +	-	-
Blood bands	+	+	+	+ -	+ -	+ -	- +	-	-

+ = all bands present at full strength: $\pm$ = about 25% of the bands missing, the remaining bands weak: $\mp$ = about 75% of the bands missing, the remaining bands very weak: - = no bands present at all.

Chemical enzyme inhibitor

Pooled cell contents of 4 strains were electrophoresed and gel strips prepared. Each strip was pre-incubated in a concentration of the inhibitor and then transferred to the staining solution or the agar overlay block, containing the same concentration of inhibitor and incubated for the appropriate time. The concentrations of potassium cyanide used were 1M, 10^{-1}M, 10^{-2}M and 10^{-3}M. Control strips, incubated in the appropriate solutions or with the agar, were always compared with the test strips to make sure the various methods were able to produce patterns. The results are expressed below in Table 46.

Table 46

The effect of the enzyme inhibitor potassium cyanide on agents that produce patterns in gel slices

Pattern	Lowest concentration to completely inhibit
High running bands (Peroxidase Method 2)	10^{-2}M KCN
High running bands (Peroxidase Method 1)	not inhibited by 1M KCN
Low running bands (Peroxidase Method 1)	not inhibited by 1M KCN
Low running bands (Catalase Detection Method 1)	10^{-1}M KCN
Blood bands	10^{-1}M KCN

The high running brown peroxidase bands (Peroxidase Method 2) are the least sensitive to heat but the most sensitive to potassium cyanide. Despite the inhibition of the bands by 10^{-2}M KCN, the very high heat resistance would cause any modern biochemist to doubt that these brown bands were enzymic in origin. Nevertheless, there is evidence in the literature to show that some bacterial peroxidases are extraordinarily heat resistant. Callow (1926) found that even after boiling cells of Staphylococcus aureus for 1 hour, peroxidase activity was detected in the test tube. Similarly Farrell (1935) and Edwards & Rettger (1937) reported that other bacteria contain peroxidases resistant to autoclaving. Since, however, no enzyme kinetics were examined and the only proof of enzymic peroxidase activity (as in my experiments) was the production

of dyes from various organic compounds as guaiacum in vitro, further experimental work is required before there is conclusive evidence that Gram positive cocci produce enzymic peroxidases which are resistant to autoclaving.

The heat resistances of the agents producing patterns by Peroxidase Method 1, Catalase Detection Method and Blood Band Detection Method are typical of those of enzymes. The appearance of the high running and the low running Peroxidase Method 1 bands, however, after incubation in 1M cyanide, indicates that if they are enzymic in origin the enzymes producing these bands are not structurally related to the known peroxidases or catalases, which are inhibited by cyanide, since this inhibitor combines with the iron molecules of these enzymes. Nevertheless, according to Farrell (1935), who studied the respiratory mechanisms of streptococci some of these bacteria could grow in 0.75% KCN and still produce a heat resistant peroxidase enzyme; he suggested that the peroxidase did not contain any iron and therefore the enzyme would not be inhibited by cyanide. Thus, it is not unlikely that the isozyme, detected by Peroxidase Method 1 are peroxidases and/or catalases, which lack any iron in their structure. From the results of the heat sensitivity and inhibition experiments, therefore, it is possible that all the agents examined in Tables 45 and 47 are enzymic in nature, although it cannot be proved conclusively from these experiments.

COMPARISON OF BANDS

The E_f values of the bands produced, the heat resistance results and the sensitivity to cyanide results were compared to see if any of the patterns detected by different staining methods were produced by the same enzyme.

a) The relationship between high running bands produced by Peroxidase Methods 1 and 2 and those produced by Succinic Dehydrogenase Method 1 and the Monamine Oxidase Method

High running bands detected by Peroxidase Method 1 have the same E_f values as those of bands detected by Peroxidase Method 2, Succinic Dehydrogenase Method 1, and Monamine Oxidase Method, but only agents capable of producing bands in the last 3 methods are heat resistant (Table 45, page 243, and page 251). The sensitivities of these heat resistant agents to the 3 chemical inhibitors is summarised in Table 47.

Table 47

Differentiation of 3 heat resistant agents capable of producing bands on gel slices by 3 chemical inhibitors

Pattern	Inhibition by		
	10^{-2}M KCN	10^{-2}M malonate	3M urea
brown bands (Peroxidase Method 2)	complete	slight (about 25%)	none
opaque bands (Succinic Dehydrogenase Method 1)	complete	complete	complete
opaque bands (Monamine Oxidase Method)	none	partial (about 50%)	complete

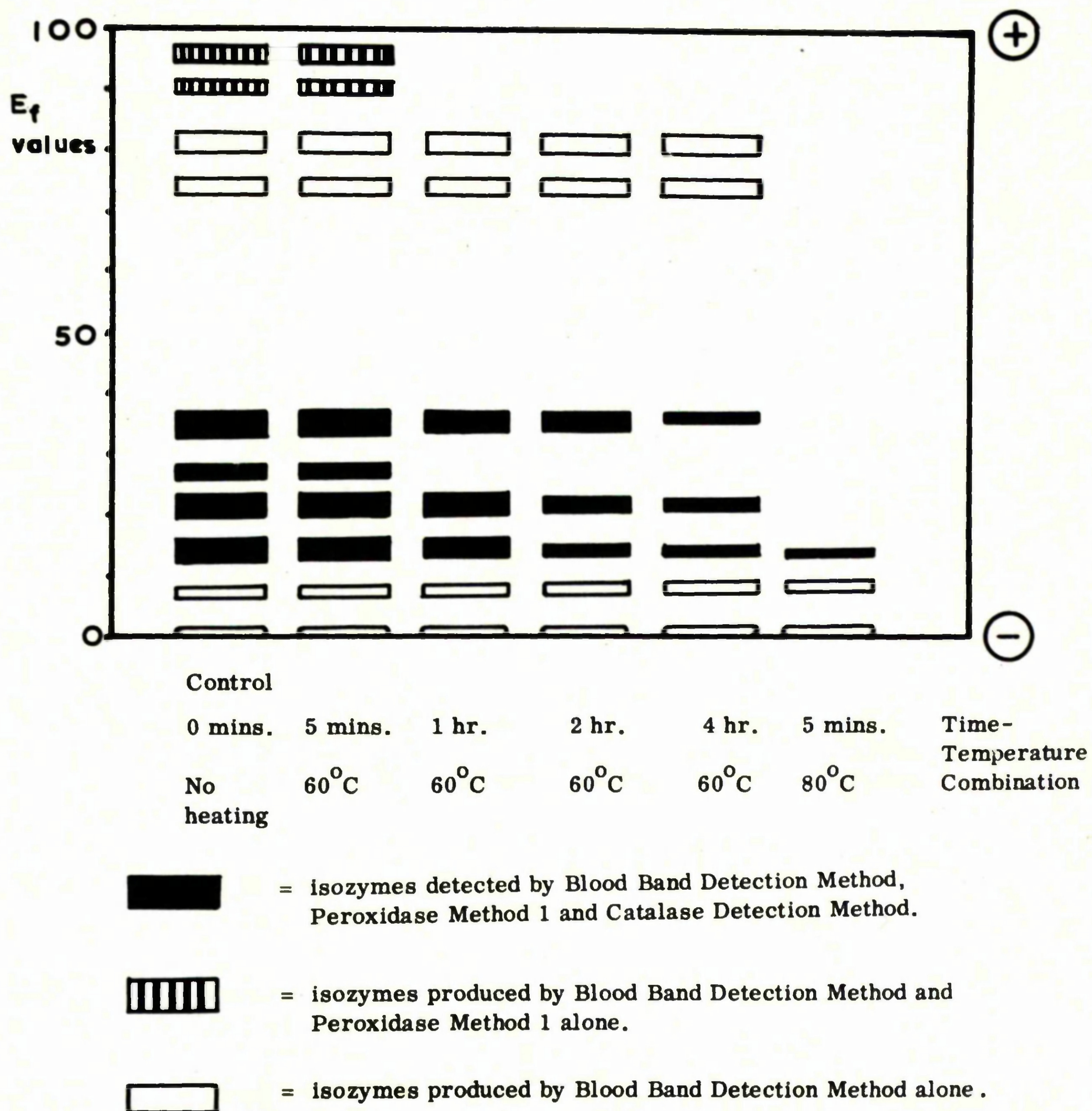
Clearly the agents responsible for the production of bands in the 4 methods examined are all different as far as enzyme inhibitors and heat resistance is concerned.

b) <u>The relationship between bands produced by Peroxidase Method 1, Catalase Detection Method and Blood Band Detection Method</u>

The agents producing patterns of low running bands with the same R_f values by Peroxidase Method 1, Catalase Detection Method or Blood Band Detection Method, have the same heat sensitivity but not the same resistance to cyanide (Tables 45 and 46). To show the relationship between bands more clearly, Diagram VII has been drawn to show the effect of heat on the production of different isozyme patterns of the pooled contents of 4 strains (page 221). The effect of heat on each isozyme is shown in the diagram (the weakest of the blood band isozymes are not included, so that the diagram remains clear). From this diagram it can be seen that the positions of the isozymes and their heat sensitivities for the low running bands of Peroxidase Method 1, catalase bands, and the strongest of the low running blood bands are identical. Similarly, the positions and the heat sensitivizy of the 2 high running bands as detected by Peroxidase Method 1 and Blood Band Detection Method are also identical. In addition, it was found that a comparison of the positions of bands in gel slices as detected by each of these 3 methods with those detected by Protein Stain indicated that each of the isozyme bands is caused by an agent protein in nature.

Diagram VII

The effect of heating on the production of isozyme bands as detected by Peroxidase Method 1, Catalase Detection Method and Blood Band Detection Method



From these results it can be concluded that a) low running bands detected by Catalase Detection Method and Blood Band Detection Method are caused by the same enzyme system, b) the catalase blood band isozymes have the same mobility as the low running bands detected by Peroxidase Method 1 but they have a different sensitivity to cyanide: thus it seems likely that there are two protein-linked enzyme sites, catalase-blood band and peroxidase, in each of the bands, c) similarly it would seem that enzymes causing the high running bands detected by Peroxide Method 1 and the 2 highest blood bands, although different, are also linked since they have the same mobilities, but not the same cyanide sensitivity.

SUMMARY

Cell contents of micrococcal and staphylococcal strains were electrophoresed on acrylamide gels, and examined for 14 different enzyme systems by a total of 28 different methods. Apparently many of the methods capable of detecting mammalian enzymes in tissue sections are not suitable for detecting bacterial isozymes in acrylamide gels, since a number of these experimental methods failed to show any isozyme bands. Nevertheless, the following isozymes were shown to occur in at least some of the micrococci and staphylococci:-

Esterases, phosphatases, β-glucuronidases, proteolytic isozymes, ureases, tributyrinases, lactic dehydrogenases, malic dehydrogenases,

peroxidases, catalases, cytochrome oxidases, starch hydrolysing isozymes, and blood band isozymes. In addition, the 3 heat resistant agents, detected by Succinic Dehydrogenase Method 1, Peroxidase Method 2, and the Monamine Oxidase Method, were present in the cell contents of all the micrococcal and staphylococcal strains examined, but there are doubts as to whether they are enxymes or not. Only esterases, catalases, and peroxidases have been previously detected in starch or acrylamide gel slices, containing electrophoresed cell contents of bacteria.

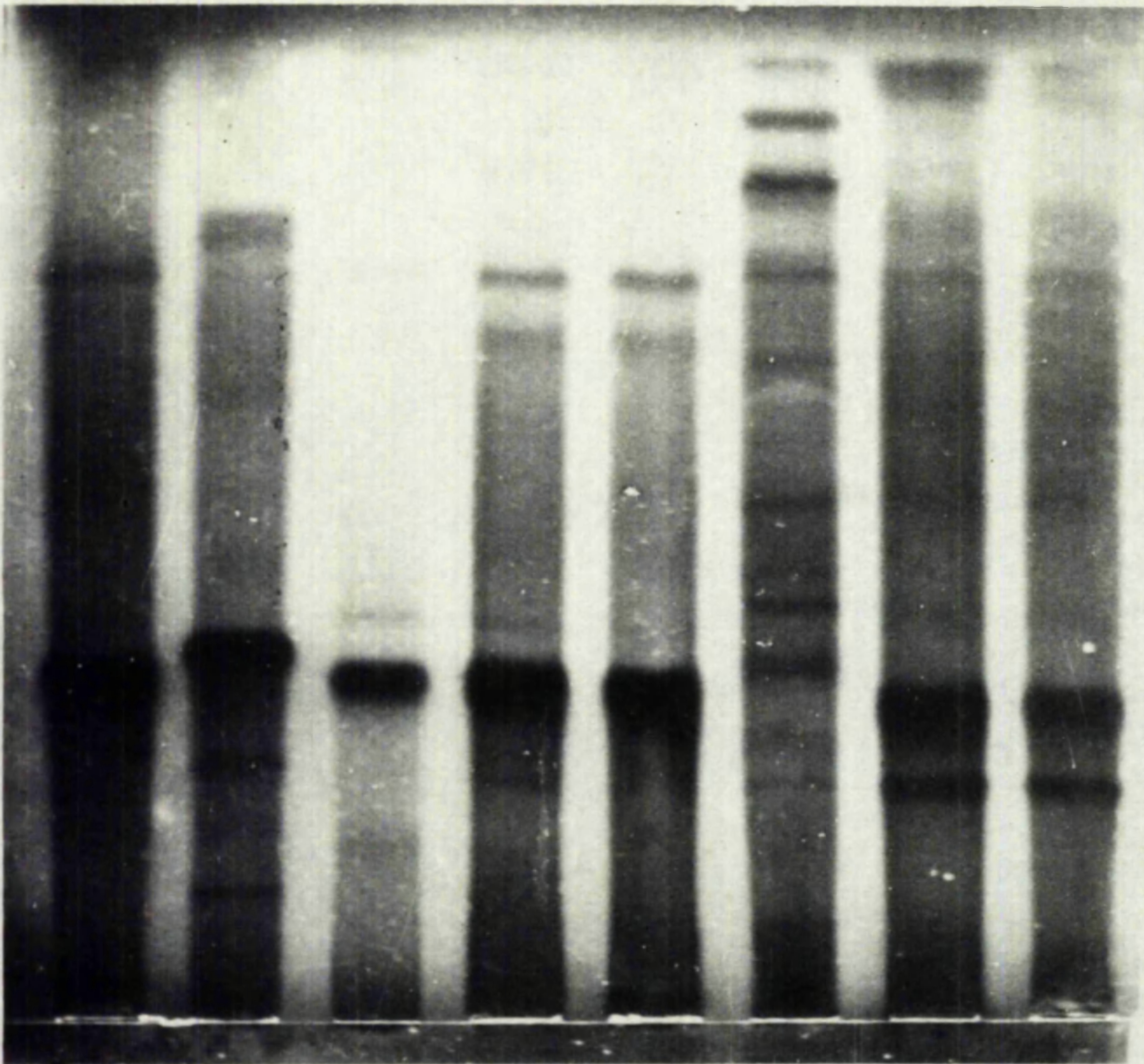
It was shown for the first time that esterase, phosphatase, β-gluouronidase, and proteolytic isozyme bands are probably caused by one enzyme system, that lactic and malic dehydrogenases are probably caused by another, and catalase bands and low running blood bands are produced by the same enzyme system, at least for micrococci and staphylococci. In addition, for the first time, these bacterial esterases, catalases, peroxidases, starch hydrolysing isozymes, blood band isozymes, and the 3 heat resistant agents were characterised by resistance to heat and resistance to chemical inhibitors.

Three different enzyme systems - esterase, blood band, and starch hydrolysing isozymes - were used as characters in an electrophoretic classification scheme. These 3 systems, and no other, were used, because these isozymes could be detected by methods certain to work, and the results were reproducible. All 406 strains of micrococci

Plate 9

Acrylamide gel slice showing blood bands

+



−

357 358 361 362 363 364 365 366 Strain numbers

Electrophoresed cell contents of 8 strains from animal skin, milk, sea water and guts of salt water fish.

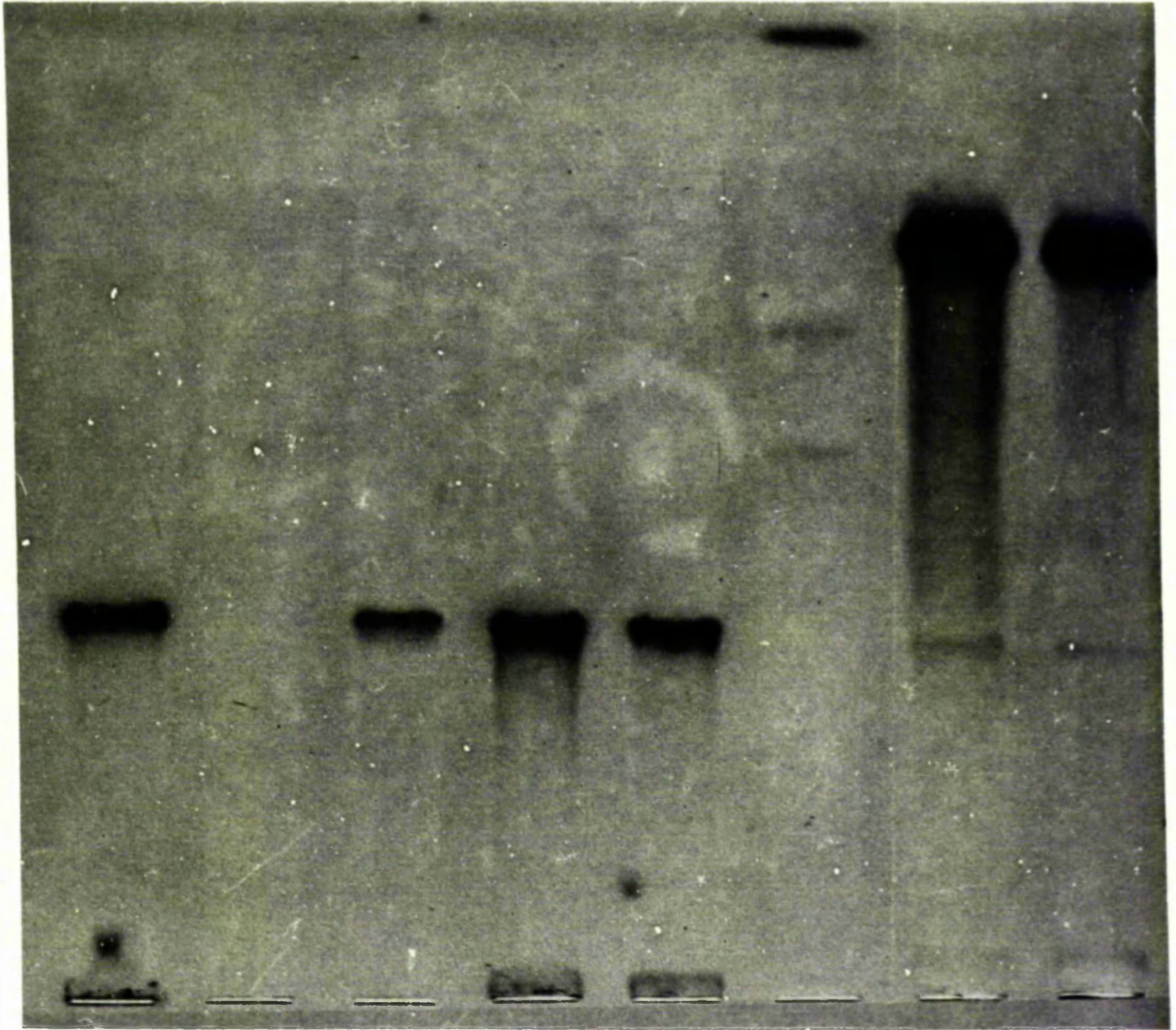
The blood band patterns of strain nos. 357, 362 and 363 are the same; similarly strain nos. 365 and 366 have identical patterns. The other 3 strains have different patterns. Note the relationship of blood band patterns and esterase band patterns (Plate 10).

The photograph was taken with transmitted light against a dark background.

Plate 10

Acrylamide gel slice stained by
Esterase Method 1 showing esterase isozyme bands

+



−

Strain numbers	357	358	361	362	363	364	365	366

Electrophoresed cell contents of the same strains shown in Plate 9. The esterase band patterns of strain nos. 357, 362 and 363 are the same; similarly strain nos. 365 and 366 have identical patterns. Strain no. 358 produces no esterase bands and strain nos. 361 and 364 produce esterase band patterns diferent from each other and the other 6 strains. Esterase band patterns are complementary to blood band patterns. (Plate 9).

and staphylococci were examined for esterase and blood band isozymes, and 144 strains for starch hydrolysing isozymes to be used as characters in the electrophoretic classification scheme, presented in the following section (page 277).

SECTION B

THE ADANSONIAN CLASSIFICATION SCHEME OF MICROCOCCI AND STAPHYLOCOCCI BASED ON ELECTROPHORETIC CHARACTERS - SCHEME 2

THE ELECTROPHORETIC CLASSIFICATION SCHEME - AN ADANSONIAN CLASSIFICATION OF THE 406 STRAINS OF MICROCOCCI AND STAPHYLOCOCCI BASED ON ELECTROPHORETIC CHARACTERS - ESTERASE, BLOOD BAND, AND STARCH HYDROLYSING ISOZYMES

Introduction

Three isozyme systems were chosen for use in an electrophoretic classification scheme of micrococci and staphylococci (page 275). Each of the 406 strains produced between a minimum of 0 and a maximum of 6 of a total of 87 esterase bands, and between a minimum of 0 and a maximum of 7 of a total of 40 blood bands. A selection of 144/406 strains produced between a minimum of 0 and a maximum of 3 starch hydrolysing isozyme bands; it was found that of the 144 strains, 5 strains which did not hydrolyse starch in agar plates produced starch hydrolysing isozyme bands (page 253), and since none of the remaining 262/406 strains hydrolysed starch in the plate, I assumed that none or very few of these 262 strains would produce starch hydrolysing isozyme bands, especially as 3/5 of the exceptions were strains of *Micrococcus luteus*, and I examined all *M. luteus* strains for these 2 starch characters; another exception was *M. radiodurans*, a strain now considered to be outwith the family *Micrococcaceae* (Baird-Parker, 1965a). Therefore, it was assumed in the electrophoretic classification scheme that all the strains producing starch hydrolysing isozyme bands were among the 144 strains examined (Table 42).

Electrophoretic characters are well suited to an Adansonian

classification, since each isozyme band can be given equal weight for classification purposes (Sokal & Sneath, 1963; page 46). The 406 strains were classified with the electrophoretic characters according to the Adansonian principle, and the results of the esterase, blood band, and starch hydrolysing isozyme analyses of the 406 strains are presented in Scheme 2 (pocket of back cover).

In Scheme 2 each of the 3 isozyme systems is assigned a different symbol. The combinations of electrophoretic characters for all 406 strains are shown (blank spaces indicate an absence of a character), and treating each character as equal it was possible to classify the strains. Since this is a comprehensive Adansonian classification scheme, I tried to classify all 406 strains, including strains with elliptically shaped cells (page 116), and catalase negative strains - I hoped that the Adansonian scheme would show whether these strains were closely or distantly related to the catalase positive cocci.

The grouping of the 406 strains is an unenviable task, since each group is created arbitrarily, even though the classification is based on the Adansonian principle, and the criteria for grouping are always open to criticism. I, thus, am quite aware that the groupings are my own personal choice, and that other taxonomists could, from the same results, group the strains differently. I have, nevertheless, tried to create groups with the minimum of bias, and present as objective a classification as possible without the aid of a computer.

Each grouping is based on a visual comparison of the arrangement of

patterns of esterase, blood band, and starch hydrolysing isozyme bands shown in Scheme 2. I consider that there are 30 Electrophoretic Groups, each containing between 1 and 119 strains. The groups containing 1 or 2 strains were the most difficult to determine, since this small number cannot constitute a "natural group". I decided, however, in such cases, that if strains shared very few electrophoretic characters with other strains, it would be advisable to place these strains into separate groups; in this way any strain found in the future with similar characters to these anomalous strains could be placed into these groups.

Apart from these anomalous strains, there were other strains, which shared characters with strains in 2 or more groups, and these strains were also difficult to classify; I made no attempt to put these intermediate strains into groups or subgroups. An intermediate strain, therefore, is defined as one which does not possess enough characters to place it in one group alone, but has characters intermediate with strains present in 2 or more groups.

Apart from the electrophoretic characters, physiological characters (the 14 main characters of Scheme 1) of the 406 strains are included in Scheme 2, in order that a comparison may be made of the physiological groupings and the electrophoretic groupings.

Electrophoretic Groups

Each of the following groups is, first of all, characterised by its esterase, blood band, and starch hydrolysing isozyme patterns

(Groups 1 - 23 and 29 and 30 contain no strains producing starch hydrolysing isozymes); each of the groups is characterised by main bands, which are common to all strains within these groups. Also mentioned are the additional bands, which strains may or may not produce; these additional bands may be used to identify strains within the groups.

Secondly, a few of the standard physiological characters are given, followed by the sources of the majority of the strains. Some of the strains were isolated from human infections (Table 23). Since I did not microscopically observe clinical samples taken from the infections, it was not possible for me to decide whether these strains were pathogenic, or saprophytic contaminants, but I used the comments of the hospital bacteriologists, who examined the samples. If one of these bacteriologists considered that a strain caused a disease, I termed this strain "pathogenic"; if the strain was considered to have been present in an infection, but not proven to be the causative agent of the infection, I termed the strain "possibly pathogenic".

Next, each of the Electrophoretic Micrococcus Groups is compared with groupings of the most widely recognised morphological and physiological classification schemes of micrococci and staphylococci at present - that of Baird-Parker (1963, 1965a; Table 5).

Finally any culture collection strains are completely characterised electrophoretically. For abbreviations N.C.T.C., N.C.I.B. and C.C.M. see Table 23.

GROUP 1 (41 strains): Strains produce esterase band no. 1 and/or band no. 81 (additional bands nos. 2,3,5,6,7,8,16,20,21,64,77 or 79), and blood band no. 3 (additional band no. 5).

Physiology: Strains are white, yellow or golden pigmented, 40/41 glucose fermentative, 19/41 mannitol fermentative, 41/41 glucose oxidative, 38/41 mannitol oxidative, 30/41 phosphatase producing, 39/41 acetoin producing, and 21/41 coagulase producing.

Source: human skin and urinary tract; 20 strains from these sources and 1 strain from a rabbit's nose (all coagulase positive) were pathogenic; one other strain from human urinary tract was possibly pathogenic.

Baird-Parker groupings: *Staphylococcus* subgroup I (21 strains), *Staphylococcus* subgroup VI (9 strains); the remaining strains are not classifiable by Baird-Parker's scheme.

Culture collection strain: *Staphylococcus citreus* N.C.T.C. 7415 produces esterase band nos. 1 and 6, and blood band no. 3.

This is a heterogeneous group, containing many of the biochemically active strains, including all 21 coagulase positive strains. It may well be that coagulase producing strains should be regarded as varieties of Group 1 organisms.

It is interesting to note that 1) of the 5 strains examined no coagulase positive strain was found to produce urease isozyme bands, although most of the strains hydrolysed urea in agar (page 239), and 2) 9/10 of the coagulase positive strains, and one coagulase negative

strain of Group 1 produced lactic dehydrogenase isozyme bands of 3 different but related patterns (Patterns 2, 3 and 4: page 244). It would appear that the lack of urease isozymes and the presence of lactic dehydrogenase isozymes could be of taxonomic significance in Electrophoretic Group 1.

GROUP 2 (7 strains): Strains produce esterase band nos. 6 and 12 (additional band nos. 10 and 11) and blood band nos. 3 or 0.

Physiology: Strains are white (5 strains) or yellow pigmented (2 strains), 7/7 glucose fermentative, 1/7 mannitol fermentative, 7/7 glucose oxidative, 4/7 mannitol oxidative, 4/7 phosphatase producing and 7/7 acetoin producing.

Source: human skin and urinary tract; 2 strains from these sources were possibly pathogenic.

Baird-Parker groupings: *Staphylococcus* subgroup II (3 strains), *Staphylococcus* subgroup VI (2 strains); the remaining strains are not classifiable by Baird-Parker's scheme.

Group 2 is a relatively small homogeneous group related to some strains in Group 1. One of the strains is the catalase negative strain no. 89; its physiological characters are given in Table 36 .

GROUP 3 (22 strains): Strains produce between 3 and 6 of esterase band nos. 20 -31, and blood band nos. 3 and 4 (3 strains have either blood band no. 3 or 4).

Physiology: Strains are white pigmented, 21/22 glucose fermentative,

7/22 mannitol fermentative, 22/22 glucose oxidative, 18/22 mannitol oxidative, 12/22 phosphatase producing and 4/22 acetoin producing.

Source: human skin and urinary tract; 6 strains from these sources were possibly pathogenic.

Baird-Parker groupings: *Staphylococcus* subgroup I (4 strains; these are mannitol fermentative, phosphatase positive, but coagulase negative), *Staphylococcus* subgroup II, *Micrococcus* subgroup 5; the remaining strains are not classifiable by Baird-Parker's scheme.

This is a heterogeneous group, containing closely related biochemically active strains; strains of Group 3 differ physiologically from those of Group 1 and Group 2 mainly in that they rarely produce acetoin. Group 3 has not been previously characterised or described before.

An observation in support of Group 3 strains being closely related is that of the 9 strains which were able to hydrolyse urea in agar all 9 produced 2 urease isozyme bands of the same E_f values, but different from those of strains in other Electrophoretic Groups (page 240).

GROUP 4 (7 strains): Strains produce esterase band no. 9 (additional band nos. 12 and 50), and blood band no. 3 or nos. 2 and 15 (additional band no. 4).

Physiology: Strains are white pigmented, 5/7 glucose fermentative, 0/7 mannitol fermentative, 6/7 glucose oxidative, 6/7 mannitol oxidative, 7/7 phosphatase producing, and 0/7 acetoin producing.

Source: healthy animal skin - mouse, guinea pig, rabbit and cow.

Baird-Parker groupings: *Micrococcus* subgroup 6 (1 strain),

Micrococcus subgroup 7 (1 strain); the glucose fermentative strains are not classifiable by Baird-Parker's scheme.

Group 4 contains relatively closely related strains, physiologically, and ecologically, as well as electrophoretically; this group has not been previously characterised.

GROUP 5 (2 strains): Strains produce esterase band nos. 5, 11, 78 and 80, and blood band no. 3 or 0.
The 2 strains are quite different physiologically, although both were isolated from diseased human urinary tracts, and they could possibly be pathogenic. One strain is catalase negative, white pigmented, glucose and mannitol fermentative, and produces phosphatase and acetoin. The other strain is catalase positive, produces a brown pink pigment, and is completely inactive biochemically, except that it oxidises glucose. Neither of these strains fits into Baird-Parker's subgroups.

Although the strains are homogeneous for esterase band numbers, they show very little else in common, and I am of the opinion that Group 5 is not a natural grouping, and may well be, in the future, dropped from the classification scheme.

GROUP 6 (119 strains): Strains produce esterase band nos. 0 or 2 or 2, 7 or 2, 5, 7 or 5, 7 or 4, 5 or 3, 5 or 3 (additional band nos. 11, 83, and 84), blood band nos. 0, 1, or 5 (additional band nos. 3 and 6).

Physiology: Strains are mostly white pigmented (1 strain is yellow pigmented, and 1 strain produces a violet pigment in addition to the white pigment), 113/119 glucose fermenting, 1/119 mannitol fermenting, 119/119 glucose oxidising, 9/119 mannitol oxidising, 99/119 phosphatase producing and 118/119 acetoin producing.

Source: mainly from healthy human skin and urinary tract; 42 strains from these sources were possibly pathogenic; 5 strains were pathogenic (1 strain, Micrococcus violagabriellae, causes a chronic human skin infection).

Baird-Parker groupings: Staphylococcus subgroup II (87 strains), Staphylococcus subgroup IV or V (15 strains), Micrococcus subgroup 3 or 4 (2 strains); the remaining strains are not classifiable by Baird-Parker's scheme.

Culture collection strains: 5 Staphylococcus epidermidis strains N.C.I.B. 8558, C.C.M. 02, C.C.M. 36, C.C.M. 40, and C.C.M. 417 all produce esterase band no. 2, and blood band no. 1. Micrococcus violagabriellae N.C.T.C. 9865 produces esterase band nos. 2, 5, and 7, and blood band no. 1; this strain is equivalent to Staphylococcus epidermidis (Evans, 1957), and Baird-Parker (1965a) Staphylococcus subgroup II. I, therefore, agree with Kocur & Martinec (1963a) and Baird-Parker (1965a), that M. violagabriellae should be reclassified as a violet pigmented strain of S. epidermidis (Group 6).

Group 6 is the largest of all the electrophoretic groups, and it would appear from Scheme 2 that within this group there might be 10 or

11 possible subgroups, based mainly on patterns of esterase band numbers. These possible subgroups, however, merge into each other, and they are difficult to characterise. Strains producing blood band nos. 1 or 1 and 5, but no esterase bands, are included in Group 6, because strains with these blood band numbers are rarely found outside Group 6.

It is interesting to note that 11/13 strains which were able to hydrolyse urea in agar were each able to produce a urease isozyme band of the same R_f value (page 239). It would appear that urease isozymes could be of taxonomic significance in Electrophoretic Group 6.

GROUP 7 (10 strains): Strains produce between 1 and 4 of esterase band nos. 2, 4, 5, 18, 20 and 21, and blood band no. 4 (additional band no. 3).

Physiology: Strains are white pigmented, 8/10 glucose fermentative, 0/10 mannitol fermentative, 10/10 glucose oxidative, 2/10 mannitol oxidative, 5/10 phosphatase producing, and 10/10 acetoin producing.

Source: mainly from healthy human skin, but 4 strains were possibly pathogenic for human skin.

Baird-Parker groupings: *Staphylococcus* subgroup II (3 strains), *Staphylococcus* subgroup IV or V (5 strains); the remaining 2 strains are not classifiable by Baird-Parker's scheme.

Culture collection strain: *Staphylococcus epidermidis* C.C.M. 1577 produces esterase band nos. 4, 5, and 20, and blood band no. 4.

Strains of Group 7 are similar physiologically and ecologically to those in Group 6, but they differ from those of Group 6 in that none of the strains in Group 6 produce blood band no. 4, whereas all the

Group 9, however, and all 6 strains in the group were isolated by Dr. A.C. Baird-Parker. It is possible that strains of this group may be found mainly on pig skin, a habitat I did not isolate strains from.

Another observation in support of Group 9 being homogeneous is that all 6 strains hydrolysed urea in agar and each produced 2 urease isozymes; the E_f values of the isozymes were the same for each strain but different from those of strains in other Electrophoretic Groups (page 239).

GROUP 10 (2 strains): strains produce esterase band nos. 9 and 19, and blood band nos. 3 or 3 and 6.

Physiology: Strains are white pigmented, 0/2 glucose and mannitol fermentative, 2/2 glucose oxidative, 1/2 mannitol oxidative, 2/2 phosphatase producing, and 1/2 acetoin producing.

Source: healthy cow skin and cheese.

Baird-Parker groupings: *Micrococcus* subgroup 6 (1 strain); the other strain is not classifiable by Baird-Parker's scheme.

GROUP 11 (3 strains): Strains produce esterase band no. 19 and blood no. 3.

Physiology: Strains are white pigmented, 0/3 glucose and mannitol fermentative, 3/3 glucose oxidative, 1/3 mannitol oxidative, 3/3 phosphatase producing, and 0/3 acetoin producing.

Source: skin and fleece of healthy sheep, and crop of healthy grouse.

Baird-Parker groupings: Micrococcus subgroup 1 or 2 (2 strains), Micrococcus subgroup 6 (1 strain).

GROUP 12 (40 strains): Strains produce esterase band no. 3 and blood band nos. 3 or 3 and 6.

Physiology: Strains mostly white pigmented (11 strains yellow pigmented), 0/40 glucose and mannitol fermentative, 39/40 glucose oxidative, 29/40 mannitol oxidative, 22/40 phosphatase producing, and 8/40 acetoin producing.

Source: cheese, milk, healthy cow and mouse skin, crop of healthy grouse, sea water, healthy fish (Gadus pollachius and G. virens), guts and healthy lobster (Homarus vulgaris) guts.

Baird-Parker groupings: Micrococcus subgroup 1 or 2 (1 strain), Micrococcus subgroup 3 or 4 (1 strain), Micrococcus subgroup 5 (12 strains), Micrococcus subgroup 6 (10 strains), Micrococcus subgroup 7 (1 strain); the remaining strains are not classifiable by Baird-Parker's scheme.

Culture collection strains: 2 Staphylococcus lactis strains, C.C.M. 1400 and 1413, produce esterase band no. 3, and blood band no. 3; 2 S. lactis strains, C.C.M. 1412 and 1407 produce esterase band no. 3 and blood band nos. 3 and 6.

Group 12 is a heterogeneous group of strains, corresponding approximately to Staphylococcus lactis (page 23).

GROUP 13 (7 strains): Strains produce esterase band no. 3, and between 3 and 4 of esterase band nos. 31, 53, 54, 55, 57, 58 and 59, and blood band no. 3 (additional band nos. 4, 6 and 20).

Physiology: Strains are white pigmented, 2/7 glucose fermentative, 1/7 mannitol fermentative, 5/7 glucose fermentative, 2/7 mannitol oxidative, 6/7 phosphatase producing, and 3/7 acetoin producing.

Source: cheese.

Baird-Parker groupings: *Staphylococcus* subgroup II (1 strain), *Micrococcus* subgroup 6 (1 strain), *Micrococcus* subgroup 7 (2 strains); the remaining strains are not classifiable by Baird-Parker's scheme.

GROUP 14 (37 strains): Strains produce no esterase bands and blood band nos. 3 or 3 and 6.

Physiology: Strains mostly white pigmented (5 strains yellow pigmented), 7/37 glucose fermentative, 1/37 mannitol fermentative, 37/37 glucose oxidative, 26/37 mannitol oxidative, 28/37 phosphatase producing, and 16/37 acetoin producing.

Source: cheese, healthy human, cow, pig, rabbit, guinea pig, and fish skin, bacon and dust.

Baird-Parker groupings: *Staphylococcus* subgroup I (1 strain, mannitol fermentative, phosphatase producing, but coagulase negative), *Staphylococcus* subgroup II (2 strains), *Staphylococcus* subgroup VI (2 strains), *Micrococcus* subgroup 1 or 2 (2 strains), *Micrococcus* subgroup 3 or 4 (3 strains), *Micrococcus* subgroup 5 (1 strain),

Micrococcus subgroup 6 (14 strains); the remaining strains are unclassifiable by Baird-Parker's scheme.

Culture collection strains: Staphylococcus saprophyticus N.C.T.C. 7292 and S. lactis N.C.T.C. 189 both produce no esterase bands but blood band no. 3; S. lactis C.C.M. 1404 produces no esterase bands but blood bands nos. 3 and 6.

Group 14 is a heterogeneous group. This is understandable, since the strains in this group do not have any esterase bands to characterise them, and the blood bands, 3 or 3 and 6, are found in other groups, including both micrococci and staphylococci.

GROUP 15 (2 strains): Strains produce esterase band no. 17, and blood band no. 3.

Physiology: Strains are white pigmented, 2/2 glucose fermentative, 0/2 mannitol fermentative, 2/2 glucose oxidative, 0/2 mannitol oxidative, 0/2 phosphatase producing, and 1/2 acetoin producing.

Source: one strain from healthy mouse skin, and one strain causative of wound sepsis in a human baby.

Baird-Parker groupings: Staphylococcus subgroup IV or V (1 strain); the other strain is not classifiable by Baird-Parker's scheme.

GROUP 16 (3 strains): Strains produce esterase band no. 13, and blood band no. 3.

Physiology: Strains mostly yellow pigmented (1 strain white pigmented), 0/3 glucose and mannitol fermentative, 3/3 glucose oxidative, 3/3
0/3 glucose and mannitol fermentative, 3/3 glucose oxidative, 3/3
mannitol oxidative, 3/3 phosphatase producing, and 1/3 acetoin producing.

Source: healthy human and guinea pig skin.

Baird-Parker groupings: *Micrococcus* subgroup 6 (2 strains); the remaining strain is not classifiable by Baird-Parker's scheme.

GROUP 17 (10 strains): Strains produce esterase band no. 14 and blood band no. 3 (additional band nos. 4 and 6).

Physiology: Strains are mostly white pigmented (3 strains are yellow pigmented), 0/10 glucose and mannitol fermentative, 10/10 glucose oxidative, 5/10 mannitol oxidative, 8/10 phosphatase producing, and 3/10 acetoin producing.

Source: mainly healthy human and cow skin, but 2 strains from human skin were pathogenic and 2 other strains possibly pathogenic.

Baird-Parker groupings: *Micrococcus* subgroup 3 or 4 (1 strain), *Micrococcus* subgroup 6 (4 strains); the remaining strains are not classifiable by Baird-Parker's scheme.

GROUP 18 (4 strains): Strains produce esterase band no. 76, and either blood band no. 3 (additional band no. 6), or blood band nos. 1 and 5.

Physiology: Strains are white pigmented, 3/4 glucose fermentative, 0/4 mannitol fermentative, 4/4 glucose oxidative, 3/4 mannitol oxidative, 3/4 phosphatase producing, and 4/4 acetoin producing.

Source: human skin and urinary tract, and dust; 2 strains from skin and urine were pathogenic.

Baird-Parker groupings: *Staphylococcus* subgroup II (1 strain),

Staphylococcus subgroup VI (1 strain); the remaining stains are not classifiable by Baird-Parker's scheme.

GROUP 19 (2 strains): Strains produce esterase band nos. 15 and 70 (additional band nos. 71 and 72), and blood band no. 3 (additional band no. 15).

Physiology: Strains are either white or yellow pigmented, 1/2 glucose fermentative, 0/2 mannitol fermentative, 2/2 glucose oxidative, 0/2 mannitol oxidative, 2/2 phosphatase producing, and 1/2 acetoin producing.

Source: healthy cat and mouse skin.

Baird-Parker groupings: Staphylococcus subgroup II (1 strain); the other strain is not classifiable by Baird-Parker's scheme.

GROUP 20 (1 strain): Strain produces esterase band nos. 69 and 87, and blood band nos. 3 and 6.

Physiology: Strain is white pigmented, does not ferment either glucose or mannitol, nor does it oxidise mannitol, but oxidises glucose; produces phosphatase but not acetoin.

Source: dust .

Baird-Parker grouping: strain not classifiable by Baird-Parker's scheme.

Group 20 is a very small group; the physiology and the blood band pattern of the strain are common to other electrophoretic groups, but the esterase band pattern is unique for Group 20.

GROUP 21 (3 strains): Strains produce between 2 and 4 of esterase band nos. 11, 32, 45, 46, and 85, and blood band no. 2 (additional blood band nos. 31 and 34).

Physiology: Strains are mostly white pigmented (1 strain is yellow pigmented), 1/3 glucose fermentative, 0/3 mannitol fermentative, 2/3 glucose oxidative, 1/3 mannitol oxidative, 1/3 phosphatase producing, and 1/3 acetoin producing.

Source: healthy guinea pig skin, and 2 culture collection strains of unknown source.

Baird-Parker groupings: *Staphylococcus* subgroup VI (1 strain), *Micrococcus* subgroup 7 (1 strain); the remaining strain is not classifiable by Baird-Parker's scheme.

Culture collection strains: *Staphylococcus lactis* N.C.T.C. 7564 produces esterase band nos. 11, 45, and 46, and blood band no. 2. *Micrococcus candidus* N.C.I.B. 8610 produces esterase band nos. 45 and 85, and blood band no. 2. This strain fermented glucose and oxidised mannitol; therefore it is clearly not a micrococcus in the generally accepted terminology.

GROUP 22 (4 strains): Strains produce esterase band no. 37, and blood band nos. 3 or 3 and 6.

Physiology: Strains are mostly yellow pigmented (1 strain is white pigmented), 1/4 glucose fermentative, 0/4 mannitol fermentative, 4/4 glucose oxidative, 3/4 mannitol oxidative, 3/4 phosphatase producing, and 1/4 acetoin producing.

Source: healthy pig and rabbit skin, and dust.

There are possibly 2 subgroups within Group 22:-

a) 3 strains: strains producing esterase band no. 37, and blood band nos. 3. Strains are yellow pigmented, glucose and mannitol oxidative, and phosphatase producing. Source: pig and rabbit skin. Baird-Parker grouping: Micrococcus subgroup 6.

b) 1 strain: strain producing esterase band no. 37, and blood band nos. 3 and 6. Strain is white pigmented, glucose but not mannitol fermentative, and acetoin but not phosphatase producing. Source: dust. Baird-Parker grouping: Staphylococcus subgroup IV or V.

There are, however, not enough strains to justify subdivision at present, and it remains to be seen whether further isolates, which can be placed in Group 22, can fit into either of these possible subgroups.

GROUP 23 (3 strains): Strains produce 2 of esterase band nos. 44, 56 and 86, and blood band no. 3.

Physiology: Strains are white pigmented, 0/3 glucose and mannitol fermentative, 3/3 glucose oxidative, 2/3 mannitol oxidative, 2/3 phosphatase producing, and 1/3 acetoin producing.

Source: healthy cow skin and cheese.

Baird-Parker groupings: Micrococcus subgroup 6 (1 strain); the 2 remaining strains are not classifiable by Baird-Parker's scheme.

Culture collection strain: Micrococcus aurantiacus (Microbiology Dept., University of Glasgow, culture collection) produces esterase band nos. 44 and 56, and blood band no. 3.

GROUP 24 (27 strains): Strains produce between 0 and 4 of esterase band nos. 9, 36 - 44 and 83, between 1 and 7 of blood band nos. 3, 8 - 12, 23, 26, 29 - 40, and between 0 and 2 of starch hydrolysing enzyme band nos. 2 - 6.

Physiology: Strains are yellow pigmented, 0/27 glucose or mannitol fermentative, 1/27 glucose oxidative, 0/27 mannitol oxidative, 6/27 phosphatase producing, 3/27 acetoin producing, and 23/27 starch hydrolysing.

Source: Strains mostly from culture collections, and source, therefore, unknown, but source of 4 strains is dust, bacon, ice-cream and fish guts.

Baird-Parker groupings: Micrococcus subgroup 7 (26 strains); the remaining strain is not classifiable by Baird-Parker's scheme.

Culture collection strains: Staphylococcus afermentans N.C.T.C. 2665. produces esterase band nos. 36, 37, 41 and 44, blood band nos. 10, 29, 31, 33 and 34, and starch hydrolysing enzyme band no. 5. This species should be reclassified as Micrococcus luteus.

S. flavocyaneus N.C.T.C. 7011 produces esterase band no. 9, 36, 37 and 40, blood band nos. 10, 29, 32, 33 and 34, and starch hydrolysing enzyme band no. 5. This strain produces a black pigment, in addition to the yellow pigment typical of strains in Group 24a. This species is clearly a variety of Micrococcus luteus.

S. luteus N.C.T.C. 8512 produces no esterase bands, but blood band nos. 10, 30 and 32, and no starch hydrolysing enzyme bands.

Sarcina lutea (Microbiology Dept., University of Glasgow, culture

collection) produces esterase band nos. 36 and 39, blood band nos. 10, 11, 29, 31, 33, 34 and 35, and starch hydrolysing enzyme band no. 5. This species should be reclassified as Micrococcus luteus.

Micrococcus sodonensis N.C.I.B. 8854 produces esterase band nos. 9, 37, 40, 43 and 44, blood band nos. 10, 29, 31, 33 and 34, and starch hydrolysing enzyme band no. 5. This species is clearly a strain of Micrococcus luteus.

M. luteus C.C.M. 248 produces esterase band nos. 36 and 41, blood band nos. 10, 29, 31, 33 and 34, and starch hydrolysing enzyme band no. 5.

M. luteus C.C.M. 291 produces esterase band no. 36, blood band nos. 8, 10, 29, 31, 32, 33 and 34, and starch hydrolysing enzyme band no. 5.

M. luteus C.C.M. 310 produces esterase band nos. 36 and 38, blood band nos. 12, 31, 32 and 34, and starch hydrolysing enzyme band no. 5.

M. luteus C.C.M. 352, C.C.M. 365, and C.C.M. 531 produces esterase band no. 9, blood band nos. 10, 29, 31, 32, 33 and 34, and starch hydrolysing enzyme band no. 5.

M. luteus C.C.M. 370 produces esterase band nos. 9, 36, 40 and 42, blood band nos. 10, 29, 31, 33 and 34, and starch hydrolysing enzyme band no. 5.

M. luteus C.C.M. 410 produces esterase band nos. 36 and 39, blood band nos. 10, 29, 31 and 34, and starch hydrolysing enzyme band no. 5.

M. luteus C.C.M. 443 produces esterase band nos. 9, 36 and 44, blood band nos. 10, 29, 31, 33 and 34 and starch hydrolysing enzyme band no. 5.

M. luteus C.C.M. 658 produces esterase band nos. 9, 36, 40 and 43, blood band nos. 10, 11, 29, 31, 32, 33 and 34, and starch hydrolysing enzyme band no. 5.

M. *luteus* C.C.M. 810 produces esterase band no. 37, blood band nos. 11, 30 and 31, and starch hydrolysing enzyme band no. 5.

M. *luteus* C.C.M. 855 produces esterase band nos. 9 and 36, blood band nos. 10, 29, 30, 31, 33 and 34, and starch hydrolysing enzyme band no. 5.

M. *lysodeikticus* (Microbiology Dept., University of Glasgow, culture collection) produces esterase band nos. 36, 37, 41 and 44, blood band nos. 10, 29, 31 and 33, and starch hydrolysing enzyme band no. 5. This species is clearly a variety of *Micrococcus luteus*, which is probably very sensitive to lysozyme.

M. *conglomeratus* C.C.M. 740 produces esterase band nos. 9, 36 and 83, blood band nos. 3 and 10, and starch hydrolysing band no. 5.

M. *conglomeratus* C.C.M. 836 produces esterase band no. 36, blood band nos. 10, 23, and 26, and starch hydrolysing enzyme band no. 5.

Unlike physiological classifications, this electrophoretic classification has shown that the yellow pigmented biochemically weak micrococci belong to a heterogeneous group of strains with closely related characteristics.

There are probably 3 subgroups in Group 24:-

subgroup a (22 strains): Strains are yellow pigmented, and produce between 1 and 4 of esterase band nos. 9, 36 - 44, between 3 and 7 of blood band nos. 8, 10, 11, 12, 29 - 35, and between 0 and 1 of starch hydrolysing enzyme band no.5Source: dust, bacon, and all the culture collection strains of *Micrococcus luteus*. Baird-Parker grouping: *Micrococcus* subgroup 7.

Unfortunately urease isozymes are of little taxonomic significance in Group 24a - only 1 of the 5 strains which were able to hydrolyse urea in agar produced urease isozymes (page 240).

subgroup b (1 strain): Strain produces small light yellow colonies on agar media, and produces esterase band nos. 36, 42 and 43, blood band nos. 9, 36 - 40, and starch hydrolysing enzyme band nos. 2 and 4. Source: the guts of a lythe (*Gadus pollachius*), freshly caught off the Isle of Oronsay, Colonsay, Argyll, Scotland.
Baird-Parker grouping: *Micrococcus* subgroup 7. A strain, such as that in Group 24b, has not been previously described or classified. Although there is only 1 strain in this subgroup, this strain differs sufficiently in morphological, ecological and electrophoretic characters from other strains in Group 24 to place it in a separate subgroup.
subgroup c (3 strains): Strains produce yellow or orange yellow pigments, and 1 strain oxidises glucose; strains produce between 1 and 3 of esterase band nos. 9, 36 and 83, between 1 and 3 of blood band nos. 3, 10, 23 and 26, and between 1 and 2 of starch hydrolysing enzyme band nos. 3, 5 and 6. Source: ice-cream, and 2 culture collection strains of *M. conglomeratus*. Baird-Parker groupings: *Micrococcus* subgroup 7 (2 strains); the remaining strain unclassifiable by Baird-Parker's scheme.

The 2 *M. conglomeratus* strains are the least related strains in subgroup c. As can be seen from Scheme 2, 1 strain of Group 24 does not fit into any of the subgroups - this strain, *Staphylococcus afermentans*

N.C.T.C. 8512, is considered to be closely related to, but not included in, subgroup a.

GROUP 25 (1 strain): Strain produces esterase band nos. 31, 66, 67 and 68, blood band no. 12, and starch hydrolysing enzyme band no. 11.

Physiology: Strain produces a cinnabar brown pigment, and does not utilise glucose or mannitol, or produce phosphatase or acetoin.

Source: *Micrococcus cinnabareus*, Microbiology Dept., University of Glasgow, culture collection strain of unknown source.

Baird-Parker grouping: Strain is not classifiable by Baird-Parker's scheme.

This strain differs morphologically (page 306), physiologically, and electrophoretically from other micrococci and staphylococci, and the strain is probably not a micrococcus. I, therefore, exclude Group 25 from the genus *Micrococcus*.

GROUP 26 (1 strain): Strain produces esterase band nos. 3, 18, 44, 62 and 63, blood band no. 10, and starch hydrolysing enzyme band nos. 8 and 10.

Physiology: Strain produces a deep red pigment, different in colour from the pigments of *Micrococcus roseus*, does not ferment or oxidise either glucose or mannitol, nor does it produce phosphatase or acetoin, but is starch hydrolysing.

Source: *Micrococcus radiodurans* N.C.I.B. 9279 of unknown source.

Baird-Parker grouping: Strain is not classifiable by Baird-Parker's scheme.

Cell wall analysis of Micrococcus radiodurans by Work (1964) has shown that the cell walls of this organism contain components found in cell walls of Gram negative bacteria, and Baird-Parker (1965a) considered that M. radiodurans should probably be classified outwith the family Micrococcaceae. From the results of the electrophoretic grouping, this strain in Group 26 seems to be distantly related to Micrococcus roseus strains (Group 27), and I consider that Micrococcus radiodurans should remain in the Micrococcus genus until further evidence is found that excludes it from this genus; in addition, no taxonomist has suggested a genus to place this strain into, if it is not retained in the Micrococcus genus.

GROUP 27 (10 strains): Strains produce between 2 and 4 of esterase band nos. 3, 24, 26, 33, 34 and 35, between 3 and 7 of blood band nos. 10, 11, 15, 22 - 28, and between 0 and 3 of starch hydrolysing enzyme band nos. 1, 5, 7 and 9.

Physiology: Strains produce a rose red pigment, do not ferment or oxidise glucose or mannitol, or produce phosphatase or acetoin, but hydrolyse starch.

Source: most strains come from culture collections, and the sources are unknown, but 3 of the strains were isolated from either dust or bacon.

Baird-Parker grouping: Micrococcus subgroup 8 (10 strains).

Culture collection strains: Staphylococcus roseus N.C.T.C. 7523 produces esterase band nos. 24 and 33, blood band nos. 10, 15, 22 and 27, and

and starch hydrolysing enzyme band nos. 5 and 7.

Micrococcus roseus (Microbiology Dept., University of Glasgow, culture collection) produces esterase band nos. 33 and 35, blood band nos. 10, 15, 22, 25 and 28, and starch hydrolysing enzyme band nos. 1, 5 and 9.

M. roseus C.C.M. 146 produces esterase band nos. 24, 33 and 34, blood band nos. 10, 15, 22, 23, 26 and 27, and starch hydrolysing enzyme band nos. 5 and 7.

M. roseus C.C.M. 239 produces esterase band nos. 24 and 33, blood band nos. 10, 15, 22, 23, 26 and 27, and starch hydrolysing enzyme band no. 5.

M. roseus C.C.M. 560 produces esterase band nos. 24 and 33, blood band nos. 10, 22, 23, 26 and 27, and starch hydrolysing enzyme band no. 5.

M. roseus C.C.M. 758 produces esterase band nos. 24, 33, 34 and 35, blood band nos. 10, 15, 22, 23, 26 and 27, and starch hydrolysing enzyme band no. 5.

M. roseus C.C.M. 837 produces esterase band nos. 24 and 33, blood band nos. 10, 15, 22, 23, 26 and 27, and starch hydrolysing enzyme band no. 5.

Unlike physiological classifications, this electrophoretic classification has shown that the red pigmented, biochemically weak micrococci belong to a heterogeneous group of strains with closely related characteristics.

Urease isozyme bands unfortunately were not of taxonomic significance in Group 27 - only 2 strains hydrolysed urea in agar, but neither strain produced urease isozyme bands, and the 1 strain capable of producing bands failed to hydrolyse urea in agar (page 240).

GROUP 28 (1 strain): Strain produces no esterase band, but blood band no. 3, and starch hydrolysing enzyme band nos. 7 and 12.

Physiology: Strain is yellow pigmented, glucose fermentative, unable to utilise mannitol, or able to produce phosphatase, but produces acetoin.

Source: cheese.

Baird-Parker grouping: *Staphylococcus* subgroup IV or V.

This group is characterised mainly on the blood band and starch hydrolysing enzyme pattern.

GROUP 29 (1 strain): Strain produces no esterase bands, but blood band no. 2.

Physiology: Strain is white pigmented, not glucose and mannitol fermentative, but glucose and mannitol oxidative; strain does not produce phosphatase or acetoin.

Source: culture collection strain *Staphylococcus lactis* N.C.T.C. 7617 of unknown source.

Baird-Parker grouping: *Micrococcus* subgroup 5.

This strain is not well characterised, because of lack of electrophoretic characters, and yet the strain is sufficiently different from other strains to place it in a group of its own.

GROUP 30 (1 strain): Strain produces no esterase bands, but blood band nos. 13 and 14.

Strain is white pigmented, unable to utilise glucose and mannitol, or produce phosphatase or acetoin.

Source: culture collection strain *Sarcina ureae* N.C.T.C. 4819 of unknown source.

Baird-Parker grouping: *Micrococcus* subgroup 7.

Considerable evidence has been amassed to show that *Sarcina ureae* is more related to the *Bacillaceae* than the *Micrococcaceae* (page 18), and since this strain has no electrophoretic characters in common with any of the other strains examined, I have decided to exclude Group 30 from the genus *Micrococcus*.

SECTION B

DISCUSSION OF THE ELECTROPHORETIC CLASSIFICATION SCHEME - SCHEME 2

DISCUSSION OF THE ELECTROPHORETIC GROUPS.

In the preceding section I have characterised the 30 Electrophoretic Groups, and there is no clear cut division of the strains into 2 main groupings, equivalent to Micrococcus and Staphylococcus, by electrophoretic analysis. The electrophoretic results, like those of the morphological and physiological classification (Scheme 1), are unable to support the current opinion that micrococci and staphylococci should be placed into 2 genera, differentiated by the oxidation - fermentation test. I consider that all micrococci and staphylococci in 28 of the 30 Electrophoretic Groups, therefore, should be placed in 1 genus, namely Micrococcus; the other 2 Groups have been shown not to belong to the genus Micrococcus, and are probably outwith the family Micrococcaceae - Group 25 (page 300), and Group 30 (page 303).

I now compare my electrophoretic classification scheme with the characters of cell shape, an important morphological character, and catalase production, an important physiological character, in the classification of micrococci and staphylococci, to see if these characters should still be regarded as important for classifying these organisms.

The taxonomic significance of cell shape in the genus Micrococcus

Only 5 of the 406 strains that I classified possessed elliptically shaped cells (page 116):- 1 strain in Group 7, 1 strain in Group 12, 2 strains in Group 13 and 1 strain in Group 25.

I think that the elliptical shape of cells in 4 of these strains (those in Groups 7, 12, and 13) is of minor taxonomic importance, because all these strains share physiological, ecological and electrophoretic characters with other strains in their respective Groups. The fifth strain, Micrococcus cinnabareus, the sole representative of Group 25, differs in many characters, physiological and electrophoretic, in addition to cell shape, from the other strains examined; for this strain elliptical cell shape is probably of taxonomic significance. I agree with Shaw et al. (1951) that M. cinnabareus should be classified outwith the genus Micrococcus (page 30).

The taxonomic significance of cell shape, therefore, depends on other characters exhibited by strains of micrococci and staphylococci, and probably cell shape is of less significance than was previously thought.

The taxonomic significance of catalase production in the genus Micrococcus

Only 3 of the 406 strains that I classified were catalase negative. These 3 strains, nos.6, 31 and 89 are physiologically closely related (page 158), but are less closely related electrophoretically. Strain no.6 produces esterase band no.5 and blood band no.5 (Group 6); strain no.31 produces esterase band nos.5, 11, 78 and 80, but no blood bands (Group 5); strain no.89 produces esterase band nos. 6

and 11, but no blood bands (Group 2). It was expected that strain nos. 31 and 89 would not produce any blood bands, since they were catalase negative, but it was surprising to find that strain No.6 produced a blood band with an R_f value similar to that produced by some catalase positive strains. It is possible that this strain produced small amounts of catalase, undetectable by the addition of peroxide to colonial growth, but detectable as catalase and blood bands on acrylamide gel slices, after application of the appropriate reagents.

All the 3 catalase negative strains fit into electrophoretic *Micrococcus* groups, which also contain catalase positive strains. It would appear, therefore, from electrophoretic analysis that some apparently catalase negative strains can be classified within the genus *Micrococcus*.

Certainly, these 3 strains are not physiologically related to *Aerococcus viridans* (page 158), and Lucas & Seeley (1955), Jensen (1963), and Solomon & San Clemente (1963) have all reported the presence of catalase negative staphylococci.

Catalase production, therefore, may have less taxonomic significance in the classification of micrococci and staphylococci than was previously thought.

<u>Comparison of Electrophoretic *Micrococcus* Groups with morphological and physiological groupings of micrococci and staphylococci by other taxonomists</u>

At this stage it seemed reasonable to compare my electrophoretic classification scheme with recently published morphological and physiological classification schemes of micrococci and staphylococci. It is difficult, however, to compare classification schemes, when different criteria for classification are used in the different schemes. For instance, it is virtually impossible to compare some of Shaw *et al.* (1951) *Staphylococcus* subgroups with the Electrophoretic *Micrococcus* Groups, because these authors did not differentiate between glucose oxidation and glucose fermentation; in addition, it is just as difficult to compare Pohja (1960) subgroups with the Electrophoretic Groups of the scheme presented in this thesis, because Pohja (1960) used characters such as acid from arabinose, and nitrate reduction as major criteria for classifying some of his strains, whereas I did not examine my 406 strains for these characters.

I, therefore, restrict the comparisons to ones which can be justifiably made, and I have selected for comparison 2 classification schemes, which are probably the most frequently used by bacteriologists at present for classifying micrococci and staphylococci:-

1) the scheme in Bergey, 1957 (Breed, 1943, 1955; Smit, 1949; Hucker, 1954; Breed, 1954; Evans, 1955).

2) the scheme of Baird-Parker (1963, 1965a) .

Comparisons of these schemes with the Electrophoretic Groups are only approximate.

1) the scheme in Bergey (1957)

the species in Bergey (1957)		Electrophoretic Groups
Staphylococcus aureus	≡	Coagulase producing and mannitol fermenting strains of Micrococcus Group 1
Staphylococcus epidermidis	≡	Micrococcus Group 6
Micrococcus luteus	≡	yellow pigmented strains of Micrococcus Groups 14, 16, 17 and 22
Sarcina lutea	≡	Micrococcus Group 24
Micrococcus roseus	≡	Micrococcus Group 27

2) the scheme of Baird-Parker (1963,1965a)

Detailed comparisons are made between Baird-Parker (1963,1965a) groupings and the Electrophoretic Groups from page 281 to page 304.

Baird-Parker (1963, 1965a) groupings		Electrophoretic Groups
Staphylococcus subgroup I	≡	Micrococcus Group 1 strains positive in 3 or the following physiological characters: coagulase production, phosphatase production, mannitol fermentation.
Staphylococcus subgroup II	≡	most strains in Micrococcus Group 6

Staphylococcus subgroup III ≡ *Micrococcus* Group 9

Staphylococcus subgroups IV and V ≡ some strains in *Micrococcus* Group 6 and *Micrococcus* Group 7.

Staphylococcus subgroup VI ≡ few strains in each of the following *Micrococcus* Groups 1, 6, 14, 18, and 21.

Micrococcus subgroups 1 and 2 ≡ some strains in *Micrococcus* Group 8.

Micrococcus subgroups 3 and 4 ≡ few strains in *Micrococcus* Group 14.

Micrococcus subgroup 5 ≡ some strains in *Micrococcus* Groups 12 and 29.

Micrococcus subgroup 6 ≡ few strains in each of the following *Micrococcus* Groups: 12, 14, 16, 17, 22 and 23.

Micrococcus subgroup 7 ≡ *Micrococcus* Group 24

Micrococcus subgroup 8 ≡ *Micrococcus* Group 27

The comparisons of the 2 physiological classification schemes with the Electrophoretic Groups show that physiological and electrophoretic classification schemes of micrococci and staphylococci are only complementary for certain groups of strains, viz. *Staphylococcus epidermidis* ≡ *Staphylococcus* subgroup II ≡ *Micrococcus* Group 6.

Sarcina lutea = Micrococcus subgroup 7 = Micrococcus Group 24.

Micrococcus roseus = Micrococcus subgroup 8 = Micrococcus Group 27.

Close correlation was not found between other electrophoretic and physiological groupings.

Comparison of ecological characters with the Electrophoretic Groups

The sources of strains in the 30 Electrophoretic Groups are given from page 281 to page 304. There are few Electrophoretic Groups in which ecological characters correlate well with electrophoretic characters, but the following observations are worth making:-

1) Electrophoretic Micrococcus Groups 1 (only the coagulase positive strains), 6, 15, 17 and 18 contained strains (31 in all), which are considered by bacteriologists in various British hospitals to be pathogenic for humans. The same bacteriologists considered that strains (57 in all) from Electrophoretic Micrococcus Groups 1, 2, 3, 6, 7 and 17 were possibly pathogenic, i.e. these strains were isolated from infections, but were not proven to be the cause of the infection. It would appear that strains of Gram positive, catalase positive cocci, other than coagulase positive strains, are capable of causing disease. It is worth pointing out that of the 10 strains in Group 17, all incapable of fermenting glucose, 2 strains caused disease, and 2 additional strains were possibly responsible for urinary infections; as far as I am aware this is the first time that Gram positive, catalase positive cocci incapable of fermenting glucose, have been recorded as capable of causing an infection.

2) the 7 strains from *Micrococcus* Group 4 were all isolated from healthy animal skin (page 283).

3) it is possible that *Micrococcus* Group 9 strains may be found only on pig skin, or on the skin of humans, working with these animals, since none of the habitats, including many mammalian skins, that I isolated strains from, supported a bacterial flora containing strains of this Group (page 287).

4) it seems that strains from *Micrococcus* Groups 10, 11, 12, 13 and 14 occur in much the same kind of habitat - cheese, in or on warm or cold blooded animals, but rarely on human skin or urinary tract.

5) the 2 strains from the skin of 2 budgerigars occur in the same group (*Micrococcus* Group 6), which comprises the majority of strains isolated from human skin. It is possible, therefore, that the budgerigars obtained their skin bacterial flora from pet handlers. The 3 bacterial strains isolated from 2 domestic cats, however, belonged to different Groups (Groups 6, 19 and an intermediate strain between Groups 1 and 2), and of these only Group 6 is associated with human skin strains.

6) No strains from *Micrococcus* Groups 24 and 27 were isolated from human or animal habitats.

Comparison of Electrophoretic *Micrococcus* Groups with published classification schemes based on DNA base ratios.

The use of DNA base ratios for bacterial taxonomic purposes is comparatively new (page 78), and only in a few of the culture collection strains that I classified on morphological, physiological and electrophoretic characters are the DNA base ratios known. Yet the comparison of the DNA base ratios of these few strains with Electrophoretic *Micrococcus* Groups and Baird-Parker's (1963, 1965a) morphological and physiological subgroups yields interesting results. Table 48 shows the comparisons. In general, each of the Electrophoretic *Micrococcus* Groups, and Baird-Parker (1963, 1965a) subgroups contains strains with approximately the same %GC DNA base composition, but there is a much greater degree of similarity between the grouping by DNA base ratios and the Electrophoretic *Micrococcus* Groups, than with Baird-Parker (1963, 1965a) subgroups. The only electrophoretic group, which does not contain strains with similar DNA base ratios, is *Micrococcus* Group 24c, and, as I have pointed out on page 299, the 2 *Micrococcus conglomeratus* strains in this subgroup are physiologically and electrophoretically less related than the shared specific name would suggest.

These comparisons show that the grouping based on DNA base ratios is in greater harmony, for the limited number of Gram positive, catalase positive cocci compared, with the Electrophoretic *Micrococcus* Groups, than with Baird-Parker's (1963, 1965a) morphological and physiological subgroups.

Table 48

Comparison of Electrophoretic *Micrococcus* Groups with classification schemes based on DNA base ratios

Species	Strain no.	DNA base composition % GC	Reference	Baird-Parker (1963,1965a) subgroups	Electrophoretic *Micrococcus* Groups
Staphylococcus saprophyticus	N.C.T.C. 7292	31.5%	b	*Micrococcus* subgroup 3 or 4	Group 14
Staphylococcus lactis	N.C.T.C. 189	32%	b	*Micrococcus* subgroup 6	Group 14
Micrococcus conglomeratus	C.C.M.740	53.5%	c	*Micrococcus* subgroup 7	Group 24c
Micrococcus conglomeratus	C.C.M.836	64.0%	c	*Micrococcus* subgroup 7	Group 24c
Micrococcus radiodurans	N.C.I.B. 9279	66.0%	c	not classifiable	Group 26
Micrococcus candidus	N.C.I.B. 8610	68%	a	*Staphylococcus* subgroup VI	Group 21
Staphylococcus lactis	N.C.T.C. 7564	69%	b	not classifiable	Group 21
Micrococcus luteus	C.C.M.810	66.3%	a	*Micrococcus* subgroup 7	Group 24a
Micrococcus luteus	C.C.M.310	67.5%	a	*Micrococcus* subgroup 7	Group 24a
Micrococcus luteus	C.C.M.410	70-73.5%	b	*Micrococcus* subgroup 7	Group 24a
Micrococcus flavocyaneus	N.C.T.C. 7011	70.8%	a	*Micrococcus* subgroup 7	Group 24a
Micrococcus sodonensis	N.C.I.B. 8854	71.8%	a	*Micrococcus* subgroup 7	Group 24a
Staphylococcus afermentans	N.C.T.C. 2665	73%	b	*Micrococcus* subgroup 7	Group 24a
Micrococcus luteus	C.C.M.248	73.3%	a	*Micrococcus* subgroup 7	Group 24a
Micrococcus luteus	C.C.M.855	73.3%	a	*Micrococcus* subgroup 7	Group 24a
Micrococcus roseus	C.C.M.837	70%	b	*Micrococcus* subgroup 8	Group 27
Micrococcus roseus	C.C.M.560	70-71%	b	*Micrococcus* subgroup 8	Group 27
Micrococcus roseus	C.C.M.146	72.8%	a	*Micrococcus* subgroup 8	Group 27

a = Rosypal *et al.* (1966). b = Hill (1966). c = Bohacek *et al.* (1967).

N.C.T.C. = National Collection of Type Cultures, London. C.C.M. = Czechoslovak Collection of Microorganisms, Brno.

N.C.I.B. = National Collection of Industrial Bacteria, Aberdeen.

not classifiable = I could not classify this strain with Baird-Parker's (1963,1965a) criteria.

The use of the electrophoretic classification scheme for identifying micrococci and staphylococci

As stated on page 1, identification is an integral part of taxonomy, but and identification scheme cannot be constructed until a reliable classification scheme has been proposed and accepted by the majority of taxonomists. Morphological and physiological characters, although relatively easy to detect, are unsuitable for identifying micrococci and staphylococci, since I have shown that there are few combinations of these characters (Scheme 1) typical, in my opinion, of a natural grouping. The electrophoretic classification scheme, however, has been shown to be successful in classifying the vast majority of 406 strains of micrococci and staphylococci, and an electrophoretic identification scheme based on the classification scheme should be designed. Nevertheless, it would be difficult for a bacteriologist using the design of the electrophoretic classification scheme, to identify an unknown micrococcus or staphylococcus, since the electrophoretic classification scheme is not dependent on the characters of actual R_f values of isozyme bands, but on the characters of the relative positions of the bands. In order to identify his unknown strain, the bacteriologist would require samples of cell contents containing each of the 87 esterase isozymes, 40 blood band isozymes, and 12 starch hydrolysing isozymes. Clearly it would not be easy to supply every bacteriologist, who wished to identify a micrococcus or staphylococcus, with these samples, even assuming he knew how to electrophorese the samples across acrylamide gels, and detect

esterases, blood bands and starch hydrolysing isozymes in the gels.

A more feasible plan for identifying micrococci and staphylococci by the electrophoretic classification scheme would be to have a central laboratory, in which all the strains representative of the 30 Electrophoretic Groups would be cultured and maintained, and where all the required samples of cell contents would be kept, and renewed periodically. The central laboratory should, then, be able to identify any micrococcus or staphylococcus sent to it. The unknown strain would be grown on agar, the cells harvested, and disintegrated, and the cell contents electrophoresed, along with the laboratory representative samples, in an acrylamide gel, and afterwards stained for esterases, blood bands, and starch hydrolysing enzymes. Comparison of the known enzyme bands with those produced by the unknown strain should allow this strain to be characterised electrophoretically, and placed into one of the Electrophoretic Groups.

Thus it is only in its use, and not in its construction, that the electrophoretic identification scheme would differ from the electrophoretic classification scheme.

S U M M A R Y

In the course of 3 years, 274 strains of micrococci and staphylococci were isolated from a variety of habitats, and 132 strains were obtained as pure cultures, mainly from culture collections; this gave a total of 406 strains for taxonomic studies. All 406 strains were examined for a total of 49 morphological and physiological characters, and the strains were compared for characters in common. It would found that strains with practically all combinations of characters existed, from the coagulase positive, biochemically active staphylococci, through many intermediate groupings to the biochemically weak yellow and red micrococci; a classification scheme based on a few arbitrarily chosen main characters, as is the tradition in this group of bacteria, would be entirely artificial. After discussing some of these main characters, and finding practically all of them unsuitable as important criteria for classifying the 406 strains of micrococci and staphylococci, I decided to adopt the Adansonian approach to classification, and treated 49 morphological and physiological characters as being equal. As I expected, the results of this Adansonian classification demonstrated that there was no clear division of the 406 strains into 2 main groups, equivalent to the genera Micrococcus and Staphylococcus, but there was a division into very many small groupings, the majority of which contained only one strain. The most important conclusion drawn from this scheme was that all micrococci and staphylococci should be placed into one genus - Micrococcus, and it seemed unlikely that further division of this genus into natural groupings by morphological and physiological characters

was possible.

Consequently, other characters, in particular those demonstrated by electrophoretic methods, were examined for their suitability in classification. I studied the isozyme bands, detected by specific colour reactions in acrylamide gel slices, which contained electrophoresed cell contents of the 406 micrococcal and staphylococcal bacterial strains, and I found that only 3 types of isozymes - esterase isozymes, used in classifications of other bacteria, and 2 previously unreported isozyme systems, blood band isozymes and starch hydrolysing isozymes - were of use in classifying my strains. These strains showed great variety in the number and mobility of the isozyme bands; the strains produced between 0 and 6 of 87 esterase bands between 0 and 7 of 40 blood bands, and between 0 and 3 of 12 starch hydrolysing isozyme bands. With the 139 electrophoretic characters of these 3 isozyme systems, the 406 strains were classified by Adansonian means, and it was found that all but 2 of the strains fell into a large group, the genus *Micrococcus*, and within this genus it was possible to detect natural groupings - 28 *Micrococcus* Groups. In addition, there were 2 other Electrophoretic Groups (25 and 30), which possessed electrophoretic characters, completely unrelated to the other Electrophoretic Groups, and these were excluded from the genus *Micrococcus*. The electrophoretic grouping does not compare well with my own Scheme 1, and published morphological and physiological classifications, but it does fit in closely with DNA base ratio

analysis groupings.

The classification scheme of micrococci and staphylococci, based on electrophoretic characters, seems to have certain advantages over schemes based on morphological and physiological characters, since electrophoretic analysis clearly shows the existence of natural groupings within one large group. Taxonomists have not been previously aware of the existence of Groups like *Micrococcus* Group 3, which, apart from a unique electrophoretic pattern of characters, contains strains which oxidise and ferment mannitol, and yet are coagulase negative.

An important contribution to the taxonomy of micrococci and staphylococci has been made by the creation of the electrophoretic classification scheme since

1) coagulase positive staphylococci have been shown to be closely related to certain coagulase negative strains (strains of *Micrococcus* Group 1). In addition, strains of micrococci and staphylococci, other than those producing coagulase, have been shown to cause disease. The character of coagulase production, therefore, can no longer be regarded as the sole criterion for classifying a unique group of pathogenic staphylococci, although it is a useful identification character.

2) the yellow and red pigmented biochemically weak micrococci - Electrophoretic *Micrococcus* Groups 24 and 27 respectively - have been classified on positive characters i.e. on electrophoretic characters which the strains possess, instead of the almost complete lack of

physiological characters, except pigmentation, which have been previously used to classify these organisms.

An identification scheme, based on the electrophoretic classification scheme, is proposed which would identify any unknown micrococcus or staphylococcus.

BIBLIOGRAPHY

REFERENCES

Abd-el-Malek, Y. & Gibson, T. (1948) J. Dairy Res. 15, 249.

Adanson, M. (1763) Familles des Plantes cited by P.H.A. Sneath (1957a) J. gen. Microbiol. 17, 184.

Andersen, E.K. (1943) Acta path. microbiol. scand. 20, 242.

Anderson, J.I.W. (1962) J. appl. Bact. 25, 362.

Andrewes, F.W. & Gordon, M.H. (1907) cited by S.T. Cowan (1962) J. appl. Bact. 25, 324.

Auletta, A.E. & Kennedy, E.R. (1966) J. Bact. 92, 28.

Baillie, A. & Norris, J.R. (1963) J. appl. Bact. 26, 102.

Baillie, M.A.H. (1964) Antigen Changes accompanying Spore Formation in *Bacillus cereus*. Thesis, University of Glasgow.

Baird-Parker, A.C. (1963) J. gen. Microbiol. 30, 409.

Baird-Parker, A.C. (1965a) J. gen. Microbiol. 38, 363.

Baldwin, E. (1960) Dynamic Aspects of Biochemistry. 3rd edition. Cambridge; Cambridge University Press.

Barber, M. & Kuper, S.W.A. (1951) J. Path. Bact. 63, 65.

Barker, H.A. (1955) in Bergey's Manual of Determinative Bacteriology (1957) 7th edition. Ed. by R.S. Breed, E.G.D. Murray & N.R. Smith, London; Bailliere, Tindall & Cox. Page 473.

Barrit, M.M. (1936) J. Path. Bact. 42, 441.

Batty-Smith, C.G. (1941) J. Hyg., Camb. 41, 521.

Beijerinck, M.W. (1901) cited by Bergey D.H. in Bergey's Manual of Determinative Bacteriology (1930) 3rd edition. London; Bailliere, Tindall & Cox. Page 98.

Bergey, D.H. (1923) in Bergey's Manual of Determinative
Bacteriology (1923) 1st edition. Baltimore; Williams & Wilkins.

Bergey, D.H. (1925) in Bergey's Manual of Determinative
Bacteriology (1925) 2nd edition. Baltimore; Williams & Wilkins.

Bergey, D.H. (1930) in Bergey's Manual of Determinative
Bacteriology (1930) 3rd edition. London; Bäilliere, Tindall & Cox.

Bergey, D.H. (1934) in Bergey's Manual of Determinative
Bacteriology (1934) 4th edition. London; Bäilliere, Tindall & Cox.

Bergey, D.H. (1937) in Bergey's Manual of Determinative Bacteriology
(1939) 5th edition. Ed. by D.H. Bergey, R.S. Breed, E.G.D. Murray
& A.P. Hitchens. London; Bäilliere, Tindall & Cox. Page 268.

Bohacek, J., Kocur, M. & Martinec, T. (1967) J. gen. Microbiol. 46, 369.

Breed, R.S. (1943) in Bergey's Manual of Determinative
Bacteriology (1948) 6th edition. Ed. by R.S. Breed, E.G.D. Murray
& A.P. Hitchens. Baltimore; Williams & Wilkins. Pages 248 and 285.

Breed, R.S. (1954) in Bergey's Manual of Determinative
Bacteriology (1957) 7th edition. Ed. by R.S. Breed, E.G.D. Murray
& N.R. Smith. London; Bailliere, Tindall & Cox. Page 455.

Breed, R.S. (1955) in Bergey's Manual of Determinative
Bacteriology (1957) 7th edition. Ed. by R.S. Breed, E.G.D. Murray
& N.R. Smith. London; Bailliere, Tindall & Cox. Page 454.

Breed, R.S. (1956) Int. Bull. bact. Nomencl. Taxon. 6, 35.

Brodie, J. (1957) J. clin. Path. 10, 215.

Buchanan, R.E. (1917) J. Bact. 2, 603.

Buchanan, R.E. (1930) cited by O. Rahn (1937) Cent. f. Bakt.,
II Abt. 96, 273.

Burstone, M.S. (1959a) cited by M.S. Burstone, (1962) Enzyme
Histochemistry and its Application in the Study of Neoplasms.
London; Academic Press. Page 462.

Burstone, M.S. (1960a) cited by M.S. Burstone (1962) Enzyme Histochemistry and its Application in the Study of Neoplasms. London; Academic Press. Page 462.

Burstone, M.S. (1961) cited by M.S. Burstone (1962) Enzyme Histochemistry and its Application in the Study of Neoplasms. London; Academic Press. Page 462.

Burstone, M.S. & Folk, J.E. (1956) cited by M.S. Burstone (1962) Enzyme Histochemistry and its Application in the Study of Neoplasms. London; Academic Press. Page 424.

Cadness-Graves, B., Williams, R., Harper, G.J. & Miles, A.A. (1943) Lancet 1, 736.

Callow, A.B. (1926) Biochem. J. 20, 247.

Cann, D.C. & Willox, M.E. (1965) J. appl. Bact. 28, 165.

Castellani, A. (1955) Ann. Inst. Pasteur 89, 475.

Chapman, G.H., Berens, C., Nilson, E.L. & Curcio, L.G. (1938) J. Bact. 35, 311.

Chapman, G.H., Berens, C., Peters, A., & Curcio, L. (1934) J. Bact. 28, 343.

Christensen, W.B. (1946) J. Bact. 52, 461.

Christie, R. & Keogh, E.V. (1940) J. Path. Bact. 51, 189.

Cohn, F. (1872) cited by D.H. Bergey in Bergey's Manual of Determinative Bacteriology (1930) 3rd edition. London. Bailliere, Tindall & Cox. Page 2.

Colwell, E.V. (1939) J. Bact. 37, 245.

Committee of the Society of American Bacteriologists (1917) J. Bact. 2, 505.

Coster, E. & White, H.R. (1964) J. gen. Microbiol. 37, 15.

Cowan, S.T. (1938) J. Path. Bact. 46, 31.

Cowan, S.T. (1939) J. Path. Bact. 48, 169.

Cowan, S.T. (1955) Int. Bull. bact. Nomencl. Taxon. 5, 133.

Cowan, S.T. (1956) Int. Bull. bact. Nomencl. Taxon. 6, 99.

Cowan, S.T. (1962) J. appl. Bact. 25, 324.

Cowan, S.T. (1965) J. gen. Microbiol. 39, 143.

Cowan, S.T. & Steel, K.J. (1964) J. Bact. 88, 804.

Cowan, S.T. & Steel, K.J. (1965) Manual for the Identification of Medical Bacteria. Cambridge; Cambridge University Press.

Cremer, H.-D. & Tiselius, A. (1950) Biochem. Z. 320, 273.

Cruickshank, R. (1937) J. Path. Bact. 45, 295.

Cummins, C.S. & Harris, H. (1956a) J. gen. Microbiol. 14, 583.

Cummins, C.S. & Harris, H. (1956b) Int. Bull. bact. Nomencl. Taxon. 6, 111.

Cummins, S.L. & Cumming, C.C. (1913) J.R. Army. med. Cps. 20, 499.

Cunliffe, A.C., Gillam, G.G. & Williams, R. (1943) Lancet ii, 355.

Daranyi, J. von (1927) cited by Elek, S.D. (1959) Staphylococcus pyogenes and its Relation to Disease. Edinburgh; Livingstone. Page 203.

Davison, A.L. & Baddiley, J. (1963) J. gen. Microbiol. 32, 271.

Davison, A.L. & Baddiley, J. (1964) Nature, Lond. 202, 874.

Davison, A.L., Baddiley, J., Hofstad, T., Losnegard, N. & Oeding, P. (1964) Nature, Lond. 202, 872.

Deibel, R.H. & Niven, C.F. Jr. (1960) J. Bact. 79, 175.

De Ley, J. (1959) J. gen. Microbiol. 21, 352.

De Ley, J. (1965) Publs. Fac. Sci. Univ. J.E. Purkyne, Brno K 35, 296.

Dewey, M.M. & Conklin J.L. (1960b) J. Histochem. Cytochem. 8, 347.

Douglas, H.C. (1954) in Bergey's Manual of Determinative Bacteriology (1957) 7th edition. Ed. by R.S. Breed, E.G.D. Murray & N.R. Smith, London; Bailliere, Tindall & Cox. Page 474.

Dudgeon, L.S. (1908) J. Path. Bact. 12, 242.

Duthie, E.S. (1954a) J. gen. Microbiol. 10, 427.

Duthie, E.S. (1954b) J. gen. Microbiol. 10, 437.

Eddy, B.P. (1961) J. appl. Bact. 24, 27.

Edwards, O.F. & Rettger, L.F. (1937) J. Bact. 34, 489.

Elek, S.D. (1959) *Staphylococcus pyogenes* and its Relation to Disease. Edinburgh; Livingstone.

Elek, S.D. & Levy, E. (1950b) J. Path. Bact. 62, 541.

Epstein, F. (1897) Z. Hyg. InfektKr. 24, 1.

Erhlich, P. (1885) cited by M.S. Burstone (1962) Enzyme Histochemistry and its Application in the Study of Neoplasms. London; Academic Press. Page 431.

Evans, J.B. (1947) J. Bact. 54, 266.

Evans, J.B. (1948) J. Bact. 55, 793.

Evans, J.B. (1955) in Bergey's Manual of Determinative Bacteriology (1957) 7th edition. Ed. by R.S. Breed, E.G.D. Murray & N.R. Smith. London; Bailliere, Tindall & Cox. Page 464.

Evans, J.B., Bradford, W.L. Jr. & Niven, C.F. Jr. (1955) Int. Bull. bact. Nomencl. Taxon. 5, 61.

Fairbrother, R.W. (1940) J. Path. Bact. 50, 83.

Farrell, M.A. (1935) J. Bact. 29, 411.

Fisher, A.M. (1936) Johns Hopk. Hosp. Bull. 59, 415.

Fisk, A. (1940) Brit. J. exp. Path. 21, 311.

Fisk, R.T. (1942b) J. infect. Dis. 71, 161.

Flugge, C. (1886) Die Mikroorganismen cited by D.H. Bergey in Bergey's Manual of Determinative Bacteriology (1930) 3rd edition. London;Bailliere, Tindall & Cox. Page 6.

Foster, W.D. (1962) J. Path. Bact. 83, 287.

Fowler, R.C., Coble, D.W., Kramer, N.C. & Brown, T. McP. (1963) J. Bact. 86, 1145.

Frazier, W.C. (1926) J. infect. Dis. 39, 302.

Fusillo, M.H. & Jaffurs, W.J. (1955) J. Bact. 70, 481.

Gause, G.G., Loshkavera, N.P., Zbarsky, I.B. and Gause, G.F. (1964) Nature, Lond. 203, 598.

Gibson, T. (1953) Int. Bull. bact. Nomencl. Taxon. 3, 129.

Gibson, T. (1967) Int. Bull. bact. Nomencl. Taxon. 17, 231.

Gillespie, E.H., Devenish, E.A. & Cowan, S.T. (1939) Lancet ii, 870.

Gillespie, W.A. & Alder, V.G. (1952) J. Path. Bact. 64, 187.

Glenner, G.G., Burtner, H.J., & Brown, G.W. Jr. (1957) J. Histochem. Cytochem. 5, 591.

Glenny, A.T. & Stevens, M.F. (1935) J. Path. Bact. 40, 201.

Gomori, G. (1950c) cited by M.S. Burstone (1962) Enzyme Histochemistry and its Application in the Study of Neoplasms. London; Academic Press. Page 267.

Gomori, G. (1952b) cited by M.S. Burstone (1962) Enzyme Histochemistry and its Application in the Study of Neoplasms. London; Academic Press. Pages 267, 323, 324 and 465.

Gomori, G. (1954b) cited by M.S. Burstone (1962) Enzyme Histochemistry and its Application in the Study of Neoplasms. London; Academic Press. Page 424.

Goodsir, J. (1842) Edin. med. surg. J. 57, 430.

Gordon, A.H., Keil, B., Sebasta, K., Knessl, O. & Sorm, F. (1950) cited by H.G. Kunkel & R. Trautman in Electrophoresis, Theory, Methods, and Applications. (1959) Ed. by M. Bier. London; Academic Press. Page 233.

Gregory, M. & Mabbit, L.A. (1957) J. appl. Bact. 20, 226.

Gretler, A.C., Mucciolo, P., Evans, J.B. & Niven, C.F. Jr. (1955) J. Bact. 70, 44.

Hall, I.C. (1944) in Bergey's Manual of Determinative Bacteriology (1948) 6th edition. Ed. by R.S. Breed, E.G.D. Murray & A.P. Hitchens, Baltimore; Williams & Wilkins. Page 235.

Harden, A. (1906) Proc. Roy. Soc. B 77, 424.

Harper, E.M. & Conway, N.S. (1948) J. Path. Bact. 60, 247.

Hastings, E.G. (1904) Cent.f. Bakt., II Abt., 12, 590.

Hill, J.H. & White, E.C. (1941) J. Urol. 45, 749.

Hill, L.R. (1959) J. gen. Microbiol. 20, 277.

Hill, L.R. (1966) J. gen. Microbiol. 44, 419.

Hill, L.R. Silvestri, L.G., Ihm, P., Farchi, G. & Lanciani, P. (1965) J. Bact. 89, 1393.

Hine, T.G.M. (1922) Lancet ii, 1380.

Hobbs, B.C. (1948) J. Hyg., Camb. 46, 222.

Holt, S.J. (1958) cited by A.G.E. Pearse (1960) Histochemistry, Theoretical and Applied. London; Churchill. Page 888.

Holt, S.J. & Withers, R.F.J. (1952) cited by A.G.E. Pearse (1960) Histochemistry, Theoretical and Applied. London; Churchill. Page 888.

Hucker, G.J. (1924a) Tech. Bull. N.Y. St. agric. Exp. Sta. no. 100.

Hucker, G.J. (1924b) Tech. Bull. N.Y. St. agric. Exp. Sta. no. 102.

Hucker, G.J. (1943) in Bergey's Manual of Determinative Bacteriology (1948) 6th edition. Ed. by R.S. Breed, E.G.D. Murray & A.P. Hitchens. Baltimore; Williams and Wilkins. Pages 235 and 283.

Hucker, G.J. (1954) in Bergey's Manual of Determinative Bacteriology (1957) 7th edition. Ed. by R.S. Breed, E.G.D. Murray & N.R. Smith. London; Bailliere, Tindall & Cox. Page 455.

Hucker, G.J. (1955) in Bergey's Manual of Determinative Bacteriology (1957) 7th edition. Ed. by R.S. Breed, E.G.D. Murray & N.R. Smith. London; Bailliere, Tindall & Cox. Page 466.

Hueppe (1886) cited by Bergey in Bergey's Manual of Determinative Bacteriology (1930) 3rd edition. London; Bailliere, Tindall & Cox. Page 7.

Hughes, B.P. (1961) Clin. Chim. Acta. 6, 794.

Hunter, R.L. & Markert, C.L. (1957) Science 125, 1294.

Hutchison, J.G.P. (1962) J. Path. Bact. 84, 289.

Issacs, A. & Scouller, J.M. (1948) J. Path. Bact. 60, 135.

Jacobs, S.I., Willis, A.T. & Goodburn, G.M. (1964) J. Path. Bact. 87, 151.

Janke, A. (1930) Cent. f. Bakt., II Abt., 80, 481.

Jay, J.M. (1962) Appl. Microbiol. 10, 247.

Jensen, J. (1963) Science 141, 45.

Jesson, O., Faber, V., Rosendal, K. & Eriksen, K.R. (1959a) Acta path. microbiol. scand. 47, 316.

Jones, D., Deibel, R.H. & Niven, C.F. Jr. (1963) J. Bact. 85, 62.

Josse, J., Kaiser, A.D. & Kornberg, A. (1961) J. Biol. Chem. 236, 864.

Judical Commission of the International Committee for Bacterial Nomenclature (1958) Int. Bull. bact. Nomencl. Taxon. 8, 138.

Julianelle, L.A. (1922) J. infect. Dis. 31, 256.

Julianelle, L.A. & Wieghard, C.W. (1935) J. exp. Med. 62, 11.

Keilin, D. & Hartree, E.F. (1938) Proc. Roy. Soc. B 125, 171

Kitchell, A.G. (1962) J. appl. Bact. 25, 416.

Kluyver, A.J. & Van Niel, C.B. (1936) Cent. f. Bakt., II Abt., 94, 369.

Kocur, M. & Martinec, T. (1962) A Taxonomic Study of the Genus Micrococcus. Brno; Fac. Sci. Univ. J.E. Purkyne.

Kocur, M. & Martinec, T. (1963a) J. gen. Microbiol. 32, 185.

Kocur, M. & Martinec, T. (1963b) Int. Bull. bact. Nomencl. Taxon. 13, 201.

Kocur, M. & Mortensen, N. (1967) Acta path. microbiol. scand. 71, 141.

Kocur, M., Precechtel, F. & Martinec, T. (1966) J. Path. Bact. 92, 331.

Kohn, J. (1957) Biochem. J. 65, 9P.

Kolle, W. & Otto, R. (1902) Z. Hyg. InfektKr. 41, 369.

Kovacs, N. (1956) Nature, Lond. 178, 703.

Krassilnikov, N.A. (1959) Diagnostik der Bakterien und Actinomyceten. Jena; Gustav Fischer.

Krynski, S., Kedzia, W., Becla, E. & Krasowski, A. (1962) Ann. Inst. Pasteur, 102, 231.

Lapage, S.P. & Jayaraman, M.S. (1964) J. clin. Path. 17, 117.

Lawrence, S.H., Melnick, P.J. & Weimer, H.E. (1960) Proc. Soc. Exp. Biol. and Med. 105, 572.

Lee, K.Y., Wahl, R. & Barbu, E. (1956) Ann. Inst. Pasteur 91, 212.

Lehmann, K.B. & Neumann, R.O. (1896) cited by D.H. Bergey in Bergey's Manual of Determinative Bacteriology (1930) 3rd edition. London; Bailliere, Tindall & Cox. Page 9.

Lenning, E. (1954) cited by M.S. Pohja (1960) Suom. Maataloust. Seur. Julk. no. 96, 1. Page 33.

Lillie, R.D. (1954) cited by M.S. Burstone (1962) Enzyme Histochemistry and its Application in the Study of Neoplasms. London; Academic Press. Page 464.

Loeb, L. (1903) J. med. Res. 10, 407.

Loeb, L.J., Moyer, A. & Murray, R.G.E. (1950) Can. J. Research E. 28, 212.

Lohnis, (1911) cited by R.S. Breed in Bergey's Manual of Determinative Bacteriology (1948) 6th edition. Ed. by R.S. Breed, E.G.D. Murray & N.R. Smith. London; Bailliere, Tindall & Cox. Page 289.

Lominski, I. & Arbuthnott, J.P. (1962) J. Path. Bact. 83, 515.

Lominski, I. & Grossfield, E. (1948) Brit. med. J. i, 343.

Lominski, I., Morrison, R.B. & Smith, D.D. (1955) J. gen. Microbiol. 13, 446.

Lominski, I., Smith, D.D. & Morrison, R.B. (1953) Nature, Lond. 171, 214.

Losnegard, N. & Oeding, P. (1963a) Acta path. microbiol. scand. 58, 482.

Losnegard, N. & Oeding, P. (1963b) Acta path. microbiol. scand. 58, 493.

Lowe, G.H. (1962) J. med. lab. Technol. 19, 21.

Lowenstein (1903) cited in Topley and Wilson's Principles of Bacteriology and Immunology (1964) 5th edition. Ed. by G.S. Wilson & A.A. Miles. London; Arnold. Page 86.

Lucas, P.R. & Seeley, H.W. (1955) J. Bact. 69, 231.

Lund, B.M. (1965) J. gen. Microbiol. 40, 413.

MacDonald, A. (1946) cited by M.T. Parker (1962) J. appl. Bact. 25, 389.

MacDonald, R.E. (1962) Bact. Proc. G24.

Mackie and McCartney's Handbook of Bacteriology (1960) 10th edition. Ed. by R. Cruickshank. Edinburgh; Livingstone.

Magnin (1878) cited by D.H. Bergey in Bergey's Manual of Determinative Bacteriology (1930) 3rd edition. London; Bailliere, Tindall & Cox. Page 2.

Markert, C.L. & Moller, F. (1959) Proc. Natl. Acad. Sci. U.S. 45, 753.

Mattick, A.T.R., Cheeseman, G.C., Beeridge, N.J. & Bottazzi, V. (1956) J. appl. Bact. 19, 310.

Mazanec, K. Kocur, M. & Martinec, T. (1965) J. Bact. 90, 808.

McCarthy, B.J. & Bolton, E.T. (1963). Proc. Natl. Acad. Sci. U.S. 50, 156.

McClatchy, J.K. & Rosenblum, E.D. (1963) J. Bact. 86, 1145.

McLeod, J.W. & Gordon, J. (1923) J. Path. Bact. 26, 326.

Mercier, P., Pillet, J. & Chabanier, P. (1950) Ann. Inst. Pasteur 78, 457.

Migula, W. (1900) cited by D.H. Bergey in Bergey's Manual of Determinative Bacteriology (1930) 3rd edition. London; Bailliere, Tindall & Cox. Page 11.

Morrison, J.M., Keir, H.M., Subak-Sharpe, H. & Crawford, L.V. (1967) J. gen. Virol. 1, 101.

Morrison, R.B. (1962) J. appl. Bact. 25, 432.

Mossel, D.A.A. (1962) J. Bact. 84, 1140.

Much, H. (1908) Biochem. Z. 14, 143.

Murray, K. (1962) Biochem. biophys. Acta 59, 211

Nachlas, M.M., Tsou, K.-C., De Souza, E., Cheng, C.-S. & Seligman, A.M. (1957) cited by M.S. Burstone (1962) Enzyme Histochemistry and its Application in the Study of Neoplasms. London; Academic Press. Pages 512 and 513.

Nelson, E.L. (1958a) J. exp. Med. 107, 755.

Nelson, E.L. (1958b) J. exp. Med. 107, 769.

Norris, J.R. (1962) J. gen. Microbiol. 28, vii.

Norris, J.R. (1964) J. appl. Bact. 27, 439.

Nunheimer, T.D. & Fabian, F.W. (1942) J. Bact. 44, 215.

Oda, T. Okazaki, H. & Seki. S. (1958c) cited by M.S. Burstone (1962) Enzyme Histochemistry and its Application in the Study of Neoplasms. London; Academic Press. Page 463.

Oeding, P. (1952b) Acta path. microbiol. scand. suppl. 93, 356.

Oeding, P. (1953c) Acta path. microbiol. scand. 33, 324,

Oeding, P. (1960) Bact. Rev. 24, 374.

Oeding, P. & Vogelsang, Th. A. (1954) Acta path. microbiol. scand. 34, 47.

Oeding, P. & Williams, R.E.O. (1958) J. Hyg., Camb. 56, 445.

Ogston, A. (1882) J. Anat., Lond. 17, 24.

Orla-Jensen, Ø. (1909) Cent. f. Bakt. I Abt. 22, 305.

Paclt, J. (1958) cited by M.S. Rohja (1960) Suom. Maataloust. Seur. Julk. No. 96, 1. Page 33.

Parker, M.T. (1962) J. appl. Bact. 25, 389.

Passet (1885) cited by G.J. Hucker in Bergey's Manual of Determinative Bacteriology (1948) 6th edition. Ed. by R.S. Breed, E.G.D. Murray & A.P. Hitchens. Baltimore; Williams & Wilkins. Page 242.

Paul, J. & Fottrell, P.F. (1961) Ann. N.Y. Acad. Sci. 94, 668.

Pearse, A.G.E. (1960) Histochemistry, Theoretical and Applied 2nd edition. London; Churchill.

Pike, E.B. (1962) J. appl. Bact. 25, 448.

Pohja, M.S. (1960) Suom. Maataloust. Seur. Julk. no. 96, 1.

Pohja, M.S. & Gyllenberg, H.G. (1962) J. appl. Bact. 25, 341.

Poulik, M.D. (1957) Nature, Lond. 180, 1477.

Prevot, A.R. (1933) Ann. Sci. Nat. 15, 23.

Prevot, A.R. (1940) Ann. Inst. Pasteur 64, 117.

Prevot, A.R. (1948) Manuel de Classification et de Determination des Bacteries Anaerobies 2nd edition. Paris; Masson.

Pribram, E. (1929) J. Bact. 18, 361.

Pribram, E. (1933) cited in Bergey's Manual of Determinative Bacteriology (1948) 6th edition. Ed. by R.S. Breed, E.G.D. Murray & A.P. Hitchens. Baltimore; Williams & Wilkins. Page 24.

Rahn, O. (1929) Cent. f. Bakt.,II Abt., 79, 321.

Rahn, O. (1937) Cent. f. Bakt., II Abt., 96, 273.

Raymond, S. & Weintraub, L. (1959) Science 130, 711.

Richardson, G.M. (1936) Biochem. J. 30, 2184.

Robinson, J. & Gibbons, N.E. (1952) cited by Hucker (1954) and Breed (1954) in Bergey's Manual of Determinative Bacteriology (1957) 7th edition. Ed. by R.S. Breed, E.G.D. Murray & N.R.Smith. London; Bailliere, Tindall & Cox. Page 463.

Robinson, K. (1966a) J. appl. Bact. 29, 179.

Robinson, K. (1966c) J. appl. Bact. 29, 616.

Rosenbach, F.J. (1884) cited by S.T. Cowan J. appl. Bact. 25, 324.

Rosenkast, P. & Clausen, J. (1962) Experimentia 18, 293.

Rosypal, S. & Kocur, M. (1963) Antonie van Leewenhoek 29, 313.

Rosypal, S., Rosypalova, A. & Horejs, J. (1966) J. gen. Microbiol. 44, 281.

Salton, M.R.J. & Pavlik, J.G. (1960) Biochim. biophys. Acta 39, 398.

Schroeter (1872) cited by D.H. Bergey in Bergey's Manual of Determinative Bacteriology (1930) 3rd edition. London; Bailliere, Tindall & Cox. Page 77.

Seedorf, J. (1924) cited by S.T. Cowan, J. appl. Bact. 25, 324.

Seligman, A.M., Tsou, K.-C., Rutenburg, S.H. & Cohen, R.B. (1954) J. Histochem. Cytochem. 2, 209.

Sharpe, M.E., Neave, F.K. & Reiter, B. (1962) J. appl. Bact. 25, 403.

Shaw, C., Stitt, J.M. & Cowan, S.T. (1951) J. gen. Microbiol. 5, 1010.

Silvestri, L.G. & Hill, L.R. (1965a) J. Bact. 90, 136.

Silvestri, L.G. & Hill, L.R. (1965b) Publs. Fac. Sci. Univ. J.E. Purkyne, Brno K 35, 309.

Smit, J. (1949) in Bergey's Manual of Determinative Bacteriology (1957) 7th edition. Ed. by R.S. Breed, E.G.D. Murray & N.R. Smith. London; Bailliere, Tindall & Cox. Page 467.

Smith, H.W. (1948a) J. Comp. Path. 58, 179.

Smith, H.W. (1948b) J. Hyg., Camb. 46, 74.

Smith, I. (1960) Chromatographic and Electrophoretic Techniques. vol. II Zone Electrophoresis. London; Heinemann. Page 137.

Smith, J.E. (1962) J. Comp. Path. 72, 131.

Smith, M.L. & Price, S.A. (1938b) J. Path. Bact. 47, 379.

Smithies, O. (1955) Biochem. J. 61, 629.

Smithies, O. (1959) Biochem. J. 71, 585.

Smithies, W.R., Gibbons, N.E. & Bayley, S.T. (1955) Can. J. Microbiol. 1, 605.

Sneath, P.H.A. (1957a) J. gen. Microbiol. 17, 184.

Sneath, P.H.A. (1957b) J. gen. Microbiol. 17, 201.

Sneath, P.H.A. (1962) in Microbial Classification, 12th Symposium of the Society for General Microbiology. Ed. by G.C. Ainsworth & P.H.A. Sneath. Cambridge; Cambridge University Press. Page 289.

Sneath, P.H.A. & Sokal, R.R. (1962) Nature, Lond. 193, 855.

Sokal, R.R. & Sneath, P.H.A. (1963) Principles of Numerical Taxonomy. San Francisco; Freeman.

Solomon, J.J. & San Clemente, C.L. (1963) Appl. Microbiol. 11, 36.

Sompalinsky, D. (1953) cited by D. Jones, R.H. Deibel & C.F. Niven Jr. (1963) J. Bact. 85, 62.

Steel, K.J. (1961) J. gen. Microbiol. 25, 297.

Steel, K.J. (1962b) J. appl. Bact. 25, 445.

Stuart, C.A., van Stratum, E. & Rustigan, R. (1945) J. Bact. 49, 437.

Subcommittee on Taxonomy of Staphylococci and Micrococci (1965b) Int. Bull. bact. Nomencl. Taxon. 15, 109.

Swartz, M.N. Trautner, T.A. & Kornberg, A. (1962) J. Biol. Chem. 237, 1961.

Tesic, Z.P. (1957) Int. Bull. bact. Nomencl. Taxon. 7, 117.

Thatcher, F.S. & Simon, W. (1956) Can. J. Microbiol. 2, 703.

Thatcher, F.S. & Simon, W. (1957) Int. Bull. bact. Nomencl. Taxon. 7, 21.

Thorne, C.J.R., Grossman, L.I., & Kaplan, N.O. (1963) Biochim. biophys. Acta 73, 193.

Thompson, R. & Khorazo, D. (1937) J. Bact. 34, 69.

Tiselius, A. (1937a) Trans. Farad. Soc. 33, 524.

Trevisan, (1885) cited by G.S. Hucker in Bergey's Manual of Determinative Bacteriology (1948) 6th edition. Ed. by R.S. Breed, E.G.D. Murray & A.P. Hitchens.Baltimore; Williams & Wilkins. Page 283.

Utzino, S., Imaizumi, M. & Nakayama, M. (1938) cited by S.D. Elek (1959) Staphylococcus pyogenes and its Relation to Disease. Edinburgh; Livingstone. Page 68.

Van Eseltine, W.P. (1955) Int. Bull. bact. Nomencl. Taxon. 5, 53.

Verhoeven, W. (1957) cited in Bergey's Manual of Determinative Bacteriology (1957) 7th edition. Ed. by R.S. Breed, E.G.D. Murray & N.R. Smith. London; Bailliere, Tindall & Cox. Page 463.

Voges, O. & Proskauer, B. (1898) Z. Hyg. InfektKr. 28, 20.

Watt, D. & Werkman, C.H. (1951) Arch. Biochem. 31, 383.

White, F., Rattray, E.A.S. & Davidson, D.J.G. (1962) J. Comp. Path. 72, 19.

Wieghard, C.W. & Julianelle, L.A. (1935) J. exp. Med. 62, 23.

Williams, R.E.O. & Harper, G.J. (1947) J. Path. Bact. 59, 69.

Williams, R.E.O., Hirch, A. & Cowan, S.T. (1953) J. gen. Microbiol. 8, 475.

Williams, R.E.O. & Rippon, J.E. (1952) J. Hyg., Camb. 50, 320.

Willis, A.T. & Turner, G.C. (1962) J. Path. Bact. 84, 337.

Wilson, G.S. & Atkinson, J.D. (1945) Lancet i, 647.

Winslow, C.-E.A. & Rogers, A.F. (1906) J. infect. Dis. 3, 485.

Winslow, C.-E.A., Rothberg, W. & Parsons, E.I. (1920) J. Bact. 5, 145.

Winslow, C.-E.A. & Winslow, A.R. (1908) cited by S.T. Cowan J. appl. Bact. 25, 324.

Work, E. (1964) Nature, Lond. 201, 1107.

ZoBell, C.E. (1946) Marine Microbiology. Waltham, Mass; Chronica Botanica. Page 45.

Zopf, W. (1883) cited by Bergey D.H. in Bergey's Manual of Determinative Bacteriology (1930) 3rd edition. London; Bailliere, Tindall & Cox. Page 5.

Zopf, W. (1891) cited by Bergey D.H.in Bergey's Manual of Determinative Bacteriology (1930) 3rd edition. London; Bailliere, Tindall & Cox. Page 98.

GLASGOW UNIVERSITY LIBRARY