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A STUDY OF *C. DIPHTHERIAE* AND OTHER MEMBERS OF THE GENUS *CORYNEBACTERIUM* WITH SPECIAL REFERENCE TO FERMENTATIVE ACTIVITY[1].

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INTRODUCTION.

THE greater part of the work embodied in this paper and its main conclusions were placed at the disposal of the Bacteriological Committee of the Medical Research Council in connection with the recently published monograph on Diphtheria (*Diphtheria, its Bacteriology, Pathology and Immunology*, H.M. Stationery Office, 1923). It was considered by that Committee that the important subject of the fermentative activity of *C. diphtheriae* and of diphtheroids generally, demanded renewed enquiry and the writer was deputed to undertake the investigation under the supervision of Prof. J. C. G. Ledingham, a member of that Committee.

The monograph as published contains the gist of the data collated and conclusions reached but as, during the past twelve months the opportunity has been taken of adding materially to the number of strains submitted to

[1] For this work a grant was given by the Medical Research Council on behalf of its Bacteriological Committee at whose disposal much of the data here collated was placed.

examination, it has been thought advisable to place on record this amplified report.

From Table I indicating source of material it will be seen that the additional strains (tabulated as Second Series) more than equal Series I discussed in the monograph.

Owing to the fact that the literature of the subject is fully dealt with in the Committee's monograph, it is unnecessary to refer to it here and the reader is therefore directed to the concluding chapter on "Diphtheroids" which contains ample information on this aspect of the subject.

Nature and source of material.

The nature and source of the material examined is shown in Table I. All the strains in Series II and the majority of those in Series I were personally isolated from material sent to the Diagnosis Department of the Lister Institute. The remaining strains were obtained from the National Collection of Type Cultures. In both series when the source of the swab was not stated it was

Table I.

	Total No. of strains	Source			
		Throat	Nose	Ear	Other sources
Series I. *C. diphtheriae* Virulent	51	36	5	1	9
„ II. „ „	51	40	7	4	—
Series I. *C. diphtheriae* Non-virulent	17	17	—	—	—
„ II. „ „	14	9	4	1	—
Series II. *C. diphtheriae* Virulence not tested	35	32	3	—	—
Series I. Diphtheroids	79	36	10	5	28
„ II. „	71	47	15	9	—

assumed to have come from the throat. Although clinical particulars were lacking in some cases the virulent and non-virulent strains in both series were definitely known to include strains from suspected cases of diphtheria, from convalescents, healthy carriers and contacts. In Series I the virulent strains under the heading "other sources" included representatives of Bell's three serological groups; one old stock strain, one strain isolated from a sore on a barber's hand and four strains isolated by Minett from cases of ulcerative lymphangitis in the horse. Most of the strains of *C. diphtheriae* in Series II not tested for virulence were isolated to confirm doubtful morphological diagnoses in primary mixed cultures. When the morphology in pure culture, the type of growth on agar and the fermentation reactions were those of *C. diphtheriae* the nature of the organism was considered to be sufficiently indicated to justify or otherwise the original opinion and except in special circumstances the matter was not taken further. The diphtheroids isolated from human sources other than the ear, nose and throat include strains from the eye, cerebrospinal fluid, genito-urinary tract, chest-wall, abdomen and skin. Two strains were isolated from milk, one from a cat and another from an oyster.

The securing of pure cultures.

In a study of fermentation reactions purity of culture is obviously of prime importance. In dealing with corynebacteria the granular character of the emulsion made by many strains renders simple plating a less reliable means of obtaining presumably pure cultures than it is with organisms forming more homogeneous emulsions. Further, owing to the pleomorphism of some members of the genus some contaminations may readily escape detection in smears. In isolating strains for this work the primary mixed culture was plated in the case of Series I on inspissated horse serum. In Series II Douglas' tellurium trypsin agar was used for the great majority of the strains and found excellent. Colonies from the plates were picked on to agar. On this first slope it was usually possible to find an isolated colony for transfer to a second agar slope. If satisfactory this was used for inoculation of an inspissated serum slope and for preliminary fermentation tests. Agar was used at this stage and in the inoculation of all subsequent biochemical tests because an alteration in type of growth possibly due to contamination can be more readily observed on agar than on inspissated serum or other medium giving more profuse growth. This routine proved satisfactory in the majority of strains, though in some instances a series of five or six replates was made and in all cases a very careful look-out was kept throughout the investigation for any indication of possible contaminations.

Preparation of medium used in fermentation tests.

After a preliminary survey of some of the more recent literature on the fermentation reactions of corynebacteria and in particular of *C. diphtheriae*, glucose, maltose, galactose, saccharose, lactose, mannite and dextrin were selected for use in Series I. In Series II mannite was omitted. The medium used in both series was Hiss's serum water containing 1 per cent. of the carbohydrate with litmus as an indicator. After preliminary filtration of the serum the serum water mixture was heated for 30 minutes at 100° C. before the carbohydrate was added. When the carbohydrate and indicator had been added the tubes were heated for 10–15 minutes at 100° C. on three successive days. To test the sterility the whole batch was then incubated for two days at 37° C.

It was quite realised that the use of a carbohydrate containing medium sterilised by heat was open to criticism and the pros and cons were considered before the work was begun and again very carefully in the course of the investigation. The advantage which weighed most in its favour was the practical certainty of sterility, and though possible changes in the carbohydrate due to the heating were not lost sight of it was hoped that the error from this cause would be constant and that therefore the results obtained with different batches would be comparable. With the majority of carbohydrates used this was found to be so, for though slight variations in the

degree of the reaction and the rate at which it was produced were observed in different batches these were not such as to lead to false conclusions. Unfortunately in the case of maltose and lactose the variations were more marked.

Anomalous fermentation reactions obtained with lactose.

With lactose it was found in some batches of media that *C. diphtheriae* and some diphtheroids gave a definite acid reaction lasting 3–5 days or longer which as a rule gradually passed off and in 10–14 days or less had completely disappeared. With other batches the reaction was so incomplete and transient that it might have been passed over as of no significance. Maltose gave similar results with Group IV and some of the cane-sugar fermenting diphtheroids.

In search of a possible explanation of these variations the *p*H of several batches of media prepared in test tubes and in bulk was estimated electrolytically. With the exception of one batch which was tested only after standing for some months in a cold room at 0–5° C. all the batches had a reaction of 8·6 or 8·7. The initial reaction of the medium would therefore not account for the variations met with and the question of hydrolysis was then considered. As chemical analysis was not possible in the circumstances biological methods had to be relied on. Accordingly batches of heated and filtered media prepared from the same material at the same time were tested side by side from the point of view of lactose fermentation only. This experiment was very carefully controlled from many aspects. Some of the tubes contained litmus as an indicator but the majority were prepared without an indicator, cresol red being added to these at various dates after inoculation. Without going into the results in detail this experiment showed that *C. diphtheriae* and diphtheroids behaving like it on heated lactose containing media did not ferment lactose when the media were filtered. On the other hand one small group of diphtheroids which had previously given a definite and permanent acid reaction in all batches of heated media still gave an acid reaction when the medium was filtered, though this appeared more slowly than in the heated media. The latter must be considered to be a true lactose fermentation whereas the acidity produced by *C. diphtheriae* on heated lactose containing media can scarcely be so regarded. This view receives confirmation from an experiment in which both media were tested with *B. typhosus*. Litmus was used as an indicator in this experiment also which showed that *B. typhosus* behaved like *C. diphtheriae*, *i.e.* a definite but transient acid reaction was produced in heated media containing lactose but not in filtered.

As a result of these experiments the possibility of using filtered media on a large scale was reconsidered. Accordingly batches of filtered and heated media were prepared from the same material at the same time and all the carbohydrates used in this work were tested. Before use both batches were incubated at 37° C. for two days and were apparently sterile. Inoculations were then made with two representatives of each of the biochemical groups

about to be described as well as with strains of virulent and non-virulent *C. diphtheriae*. Readings were taken at the usual intervals.

In this experiment it was soon obvious that some of the diphtheroids grew much better on the heated than the filtered media though with *C. diphtheriae* and the more free growing diphtheroids the difference in growth in the two media was much less marked. As the slower rate of growth meant delay in the production of acid it was a distinct disadvantage and moreover it was disappointing to find that though one type of anomalous reaction had been avoided others had occurred. These affected maltose and dextrin and recurred when the experiment was repeated with fresh media. Though probably due to some fault in the samples of maltose and dextrin used they have not so far been satisfactorily accounted for as only the diphtheroid inoculated could be demonstrated in the affected tubes. As but for the previous results and present controls with heated media there would have been no reason to doubt the genuineness of the reactions produced with maltose and dextrin in filtered media the possibility of drawing false conclusions seemed more serious than with heated media. As the extent of possible error with the latter was now fairly well known the results about to be recorded are those obtained with heated media corrected especially in the case of lactose by the use of filtered material.

Varying fermentability of different brands of dextrin.

One other difficulty was encountered, that being the varying fermentability of different brands of dextrin. Owing to one stock of dextrin having been used throughout the investigation of Series I this variability was only observed later in the course of routine work when a fresh stock came into use. As the first sample had proved of very considerable value in differentiating *C. diphtheriae* from the diphtheroids, with Dr Robison's help some of the chemical as well as the biological characters of several brands of dextrin were examined. The results of this examination are given in detail in the Diphtheria Monograph and it need only be said here that no definite relationship could be established between the rotatory and reducing power of the different brands and the readiness with which they were attacked by *C. diphtheriae*. An interesting point brought out especially by the examination of the less sensitive samples was that some strains of *C. diphtheriae* were more active dextrin-fermenters than others. With such samples the majority of strains gave an incomplete acid reaction but at one end of the scale were a few which readily produced acid and clot whilst at the other were a small number in which the reaction was exceptionally feeble. Because of the uniform results obtained with the brand of dextrin used in the examination of Series I it has been included in the accompanying table of reactions but it cannot at present be considered a suitable substance for biological work.

Recording the fermentation reactions.

Before discussing the reactions obtained the method of recording these may be briefly noted. The carbohydrate containing-tubes were inoculated from 24–48 hour agar slopes and readings made daily for the first three days and subsequently on 5th, 7th, 10th and 14th days. On a few occasions 21 and 28 day readings were recorded. Contrary to custom when reading litmus tubes these were examined by transmitted light from a north window. Uninoculated tubes were always incubated with the tests and used as controls. With *C. diphtheriae* and some of the diphtheroids definite though not always complete reactions were obtained in 24 hours. In the case of other diphtheroids, however, the reactions developed more slowly. With some strains this occurred with all the carbohydrates attacked but with others the delay was only observed in some of the test substances. In such cases it might be 3rd or even 5th day before the fermentation picture was complete. Even when the reaction had reached its maximum the degree of completeness was not necessarily the same in each carbohydrate. In some the reaction was limited to a colour change of the indicator whilst in others the change of colour was followed by the appearance of a coarse precipitate which might finally form a solid clot. Amongst themselves members of the same group might show slight variations in the strength of the reaction produced on individual carbohydrates but not infrequently even in detail the reactions of all members of the group were wonderfully uniform when the same batch of medium was used. The strains selected as types of the groups as well as many others have been examined at least five times with constant results. With very few exceptions all the strains in the first series have been completely tested at least twice and many have been under observation for two years or longer. Twenty-eight of the diphtheroids in Series II have been examined once only but the remainder have either been tested twice or oftener or two colonies from the same culture have been examined.

Fermentation reactions of C. diphtheriae.

The biochemical reactions of *C. diphtheriae* may be first considered. All the strains of virulent *C. diphtheriae* examined, 102 in all, gave the same reactions. Glucose, maltose and galactose were fermented. Glucose was most strongly attacked, clot being produced by all the strains. The tubes containing maltose and galactose were not invariably clotted and strains which produced clot in the one did not necessarily do so in the other. The reactions obtained with lactose and dextrin have already been alluded to at length. Cane-sugar and mannite were not fermented. Fermentation of cane-sugar by virulent strains of *C. diphtheriae* has been recorded by some writers but in this work when a virulent organism in all other respects indistinguishable from *C. diphtheriae* produced acid in cane-sugar it was always found that this reaction disappeared after replating even though in some instances no contamination could be demonstrated either microscopically or culturally.

Thirty-one strains morphologically and culturally indistinguishable from virulent *C. diphtheriae* gave exactly the same biochemical reactions but were not pathogenic for guinea-pigs. These must for the present be classed as non-virulent *C. diphtheriae*.

Table II.

Section	Group	No. of strains in group	No. of strains from nose and throat	No. of strains from ear	No. of strains from other sources	Glucose	Maltose	Galactose	Saccharose	Lactose	Mannite	Dextrin
A	IV	9	7	0	2	+	±	−	−	−	−	−
	III	19	10	2	7	+	+	−	−	−	−	−
		28	17	2	9							
	V	13	9	2	2	+	+	+	−	−	−	−
	V *a*	2	2	0	0	+	±	+	−	−	−	−
		15	11	2	2							
B	II	16	13	1	2	+	±	−	+	−	−	−
	VII	8	3	0	5	+	+	−	+	−	−	−
	I	21	13	4	4	+	±	+	+	−	−	−
	VIII	11	10	1	0	+	+	+	+	−	−	−
	VI	7	7	0	0	+	+	+	+	+	−	−
	IX	1	0	0	1	+	+	+	+	+	−	+
	X	1	1	0	0	+	±	+	+	−	+	−
		65	47	6	12							
C	XI	42	33	4	5	−	−	−	−	−	−	−
		150	108	14	28							
C. diphtheriae (virulent and non-virulent)						+	+	+	−	−	−	+
C. Pseudo-tuberculosis ovis						+	+	?	−	−	−	−
„ „ *murium*						+	+	−	+	−	−	−

\+ = acid production with or without clot.
± in maltose column = transient acid production.
− = no acid production.

Table III.

	Source and No. of the Diphtheroids in the Groups in											
	Series I				Series II				Series I and II			
Group	Total	Nose and throat	Ear	Other sources	Total	Nose and throat	Ear	Other sources	Total	Nose and throat	Ear	Other sources
I	15	10	1	4	6	3	3	—	21	13	4	4
II	11	9	0	2	5	4	1	—	16	13	1	2
III	11	3	1	7	8	7	1	—	19	10	2	7
IV	6	4	0	2	3	3	0	—	9	7	0	2
V	4	1	1	2	9	8	1	—	13	9	2	2
V*a*	0	0	0	0	2	2	0	—	2	2	0	0
VI	4	4	0	0	3	3	0	—	7	7	0	0
VII	5	0	0	5	3	3	0	—	8	3	0	5
VIII	3	3	0	0	8	7	1	—	11	10	1	0
IX	1	0	0	1	0	0	0	—	1	0	0	1
X	1	1	0	0	—	—	—	—	1	1	0	0
XI	18	11	2	5	24	22	2	—	42	33	4	5
Totals	79	46	5	28	71	62	9	—	150	108	14	28

FERMENTATION REACTION OF THE DIPHTHEROIDS.

The fermentation reactions given by 79 diphtheroids in Series I divided them into 11 groups. With the exception of Group XI the numbers given to the groups correspond roughly to the frequency with which they occurred in

Series I but the numbers in either series are of course too small to draw any conclusions as to the real incidence of the groups. Because of the variable and transient acidity already referred to produced in maltose by some strains, some of the groups are not sharply cut off from each other during the first days of the test. This applies to Groups I and VIII, II and VII, IV and III. In the first group of each pair the maltose reaction is variable in degree and always transient; in the second it is permanent and usually so strong that clot is formed. In Table II the biochemical reactions of the diphtheroid groups are given together with those of *C. diphtheriae*. In Table III the numbers in each group and the source are indicated.

As a discussion of the individual groups must involve consideration of their source, morphology and other characters it may be well here to examine these in relation not only to the diphtheroids but to *C. diphtheriae* as well.

Source in relation to fermentation reactions.

Any further reference to the source of the various strains of *C. diphtheriae* examined is unnecessary. In the case of the diphtheroids it is found that strains from the nose and throat occur in all the biochemical groups except IX. All the larger groups and some of the smaller contain in addition strains from other sources. Urogenital strains are found in groups III, IV, V, VII, XI; strains from the eye in Groups I, II and XI and from the ear in I, III, V, VIII and XI. The source of the diphtheroid therefore gives no indication of the fermentation reactions to be expected.

Indeed on 18 occasions representatives of two biochemical groups were isolated from the same material and on three occasions the same swab yielded diphtheroids of three groups. Though in such cases one of the diphtheroids not infrequently (12 out of 21) belonged to the non-fermenting Group XI other combinations occurred. For example, Group VIII was found three times with Group I and once with Group III. Groups III and VII occurred together twice. In addition to these instances of diphtheroids of different groups occurring together two swabs yielded a pigmented and non-pigmented strain of Group XI. Though in one of these cases the non-pigmented strain might possibly be regarded as a variant of the pigmented one, in the other instance the morphology and type of growth on agar of the two strains were so different that they must be regarded as different organisms.

Some of the diphtheroids were also found in association with *C. diphtheriae*. Group XI was isolated six times, Group I twice and Group II once from the same swab as *C. diphtheriae*. This cannot, however, be taken as representing the true incidence with which *C. diphtheriae* and the diphtheroids occur together; for in routine work whether the isolation is being carried out for the purpose of a virulence test or to confirm a morphological diagnosis its chief aim is accomplished when *C. diphtheriae* has been found. On the other hand when only a diphtheroid is isolated the plate is fished much more thoroughly to exclude the possibility of *C. diphtheriae* being present as well.

Growth on agar.

The agar used was that in general use in the Institute and was made from a trypsin broth digest, *p*H, being adjusted to 7·8. On this medium the growth of *C. diphtheriae,* virulent and non-virulent, was usually a moderately fine one but it varied to some extent on different batches; the extremes of variation being a fine streptococcal-like growth and a very thick one almost like *B. coli.*

The latter has been found with only one batch of medium in the course of three years and is therefore of little practical importance.

The diphtheroids appeared on the whole to be less influenced than *C. diphtheriae* by the slight variations occurring in different batches of agar, the great majority being very constant in the character of their growth on this medium. The chief types of growth noted varied on the one hand from fine to moderately fine and on the other from rather thick to very thick. These two chief types were found in almost equal numbers among 79 diphtheroids of the first series whilst in the second thick growths predominated. The majority of the biochemical groups contain representatives of both fine and thick growths though in some groups one type of growth occurred more frequently than the other. Thick growths predominated in Groups I, V and VII and fine growths in II, whilst in III, IV and XI the two types occurred in almost equal numbers. The fine growths were almost colourless and the thick ones as a rule white or creamy.

(*Pigmentation is discussed on p.* 250.)

Stickiness of growth was noted in the majority of the strains in Group V and in a few strains in other groups particularly in Group III but it was often a somewhat elusive character and in some cases might easily have been missed if not specially looked for. These growths had often though not invariably a certain superficial appearance of dryness and at times were somewhat friable.

All the strains in Group VI and VIII had dry wrinkled growths which in the majority of cases were pigmented. One strain in Group IV and another in Group VII gave a very similar type of growth except that it was slightly thicker and more heaped up than wrinkled.

From time to time variant colonies have been observed on agar cultures but up to the present time has not permitted the carrying out of careful plating experiments with these. Sometimes but not always they appeared immediately after isolation. Such of these variants as it was possible to test at all gave the same fermentation reactions as the parent culture but as reliable cultures were not obtained the tests were inconclusive. Though the growth on agar of some diphtheroids is indistinguishable from that of *C. diphtheriae* the character of the growth on this medium as prepared in this laboratory has come to be considered in the course of this work a very useful point in distinguishing some of the diphtheroids from *C. diphtheriae.* A thick growth whether moist, dry, sticky or pigmented is very unlikely to be a culture of *C. diphtheriae* and with experience less marked variations from the

typical diphtheriae like growth may excite suspicion as to the true nature of the organism.

Growth on serum.

On inspissated horse serum *C. diphtheriae* gives a good growth, in the first 18–24 hours almost colourless but later becoming definitely white or creamy. The diphtheroids on this medium showed on the whole less individuality than on agar. Many of them gave growths very similar to *C. diphtheriae* but in some the growth was always thin and in others it was somewhat dry. But as with agar no type of growth was invariably associated with one group of fermentation reactions.

Pigmentation.

This was studied only on agar and serum cultures and has been noted in 33 strains in all. In many cases it was shown equally well on both agar and serum but in some strains it was more marked and appeared more readily on agar. In some instances though very definite pigmentation was seen immediately after isolation, in subsequent subcultures pigment production was poor or only appeared at irregular intervals. With the exception of two very deep orange strains in Group XI the pigment shown has been a bright yellow often tending to an orange tint but in a few strains of a lemon shade. The incidence of the pigmented strains in the groups is shown in Table IV.

In the Diphtheria Monograph it has been pointed out that all the strains in Groups VI and VIII are pigmented. Among the additional strains placed in these groups in Series II one strain in group VI and three in Group VIII show very little pigment their colour as a rule not being more than a deep cream although their morphology and the general character of the growth apart from pigmentation is very like that of the other strains in the groups.

In the most markedly pigmented strain in Group VII the pigment is best shown on agar. The overnight growth on this medium is very fine and colourless but in three or four days becomes much thicker and shows a bright yellow pigment.

Pigmentation was not observed in any of the strains of *C. diphtheriae* examined.

Table IV.

		Source		Degree of pigmentation	
Group	No. of strains	Nose and throat	Other sources	Good, practically constant	Poor, inconstant or both
II	2	2	0	0	2
IV	3	2	1	2	1
V	1	0	1	0	1
V*a*	1	1	0	1	0
VI	7	7	0	6	1
VII	4	3	1	1	3
VIII	11	11	0	7	4
IX	1	0	1	1	0
XI	3	2	1	3	0

Growth in broth.

The broth used in this work was prepared in the same manner as that used as the basis for the agar medium. A few strains of *C. diphtheriae* grew badly on this medium but the majority gave good growths. In 48 hours the deposit was well marked, granular and distributed all over the bottom of the tube. The supernatant was granular or almost clear and a film was usually present. Most of the diphtheroids also grew well in this broth but in some cases the growth was poor. In a number of strains the growth resembled that of *C. diphtheriae* but in others, *e.g.* in Groups VI and VIII, though the general features of the growth were the same it was of a much coarser type. Still others gave a growth of a different character. The deposit though well marked was compact and confined to the centre of the bottom of the tube. The supernatant showed a general turbidity and a film might or might not be present. The character of the growth in broth therefore does not invariably distinguish the diphtheroids from *C. diphtheriae* and shows no constant relationship to their own fermentation reactions.

Morphology.

This was studied chiefly in smears made from 18–24 hour cultures on inspissated horse serum and stained with Löffler's methylene blue. Growths from agar were also examined and Gram's and Neisser's stains were used in some cases. The latter proved disappointing as a differential stain as many of the diphtheroids showed well-marked polar granules with it.

It is well known that the morphology of many corynebacteria varies with the age of the growth and the media on which they are grown, but variations in length, breadth and staining have also, not infrequently, been observed under apparently the same conditions. These variations occurred most commonly and were usually most marked immediately or soon after isolation. Occasionally the change in morphology has been so great as to throw doubt on the identity of the culture had not the biochemical and other characters remained unaltered. In these circumstances only a very general outline of the morphological types usually found will be given. In the first place these may be conveniently divided into very short forms and forms of medium length. Except in two recent batches of serum long or very long forms were not found as the predominant type in 18-hour serum cultures though they occurred on agar particularly with *C. diphtheriae*. Some of the very short strains were almost diplococcal and occasionally only the presence of a few longer or barred forms or the morphology on another medium made it possible to determine the real character of the culture. Members of these three primary morphological types may differ from each other in breadth or in the staining capacity of the protoplasm. The protoplasm may stain uniformly and deeply or it may stain lightly as a whole but show polar granules or deeply stained patches distributed regularly or irregularly throughout its length giving a barred or striated appearance. The outline of the organism may be clear cut

and definite or hazy and ill-defined. Haziness of outline may be shown by the whole organism or more commonly, especially among the diphtheroids, it is seen only at one or both ends. One or both ends may be pointed, rounded off or more or less swollen. Swelling of the ends is much more frequently seen in *C. diphtheriae* than in the diphtheroids. A large number of types all passing gradually into each other are formed by varying combinations of the features just described. Though not infrequently particularly among the diphtheroids one morphological type predominates in a given strain it is rare even among those members of the genus to find a culture composed strictly of one type.

Contrary to the experience of some workers, in this investigation it has been found that as a rule the difference between the morphology on agar and on serum of members of the genus has been that on the former the organism is longer or broader on both. On the other hand, marked variations in morphology were on the whole commoner on agar than on serum.

Doubtless the chief interest and importance of the morphology of the diphtheroids lies in their resemblance or otherwise to *C. diphtheriae*. Though certain morphological characters such as polar granules, marked irregularity of staining and clubbing are commonly associated with *C. diphtheriae* representatives of practically every morphological type may be found in cultures of this organism. Commonly several types are present in the same culture and an atypical type may even predominate. On the other hand, in a culture of a diphtheroid composed chiefly of a type not at all characteristic of *C. diphtheriae* a few individuals are often found which by themselves are indistinguishable from *C. diphtheriae*. Thus the opinion has been formed as a result of this work that the distinction between *C. diphtheriae* and other members of the genus depends at least as much on the general impression made by the smear as on the presence of individual types; the marked pleomorphism and the great irregularity of the arrangement of individuals in relation to each other in *C. diphtheriae* being often in marked contrast to the comparative uniformity of individuals and the impression of orderliness given by the smear of a diphtheroid as a whole. Whilst in pure culture and in the majority of cultures made direct from swabs sent to the laboratory for diphtheriae diagnosis *C. diphtheriae* can usually be distinguished from most diphtheroids with comparative certainty; in some cases particularly in primary mixed cultures it may be very difficult if not impossible to give a definite opinion as to the nature of the organism on morphological grounds only. It should not be forgotten that some of the most difficult problems in the morphological diagnosis of *C. diphtheriae* in primary cultures may be due to organisms which are not members of the genus corynebacterium.

In the course of this work three or four diphtheroids have been encountered which have given more or less constantly a morphological picture quite indistinguishable from that of *C. diphtheriae*. The first of these was isolated from a cat suspected of causing diphtheria in a family. The second is the mouse organsim *C. pseudotuberculosis murium*. Both of these ferment cane-sugar

and are not pathogenic to guinea-pigs. The morphology is therefore the chief point they have in common with *C. diphtheriae*. The third organism, *C. pseudotuberculosis ovis*, has not been so constant in its morphological resemblance to *C. diphtheriae* as the other two but on some occasions the resemblance has been very complete. It ferments glucose and maltose and some strains give a slight reaction with galactose. Cane-sugar is not fermented. In its biochemical reactions therefore it is more closely allied to *C. diphtheriae* than the other two organisms. In addition it is pathogenic to guinea-pigs but the control animal is not protected by an amount of diphtheria antitoxin which would be sufficient to protect against a similar dose of *C. diphtheriae*. The growth on agar is at first thin and dryish, later becoming definitely dry and wrinkled, a type of growth which has not been observed in any strain of virulent *C. diphtheriae*. There are grounds therefore for placing this organism also among the diphtheroids.

The fourth organism morphologically like *C. diphtheriae* will be considered when discussing Group XI.

Virulence of C. diphtheriae *and the diphtheroids.*

In Series I and in the majority of the strains in Series II the virulence of both *C. diphtheriae* and the diphtheroids was tested by the subcutaneous inoculation of a well-grown 48 hours broth culture. Sixteen strains of *C. diphtheriae* and six diphtheroids in Series II were tested by the intracutaneous method. In dealing with the few strains of *C. diphtheriae* which did not grow well on the ordinary broth used a good growth could always be obtained by the addition of ascitic fluid to the medium. In testing *C. diphtheriae* and the four strains of *C. pseudotuberculosis ovis* a dose of 2 c.c. was given to the test animal and 2·5 c.c. plus 200 units of antitoxin to the control. In the case of the diphtheroids the dose was 3 c.c. or 3·5 c.c. and no antitoxin controls were used. The weight of the guinea-pigs at the time of inoculation varied from 240–300 gms. Tested in this way virulent strains of *C. diphtheriae* caused death with characteristic post-mortem changes, in 24–72 hours. Deaths occurring later than this were either found to be due to some cause other than the inoculations, *e.g.* a Gaertner infection or pseudotuberculosis; or they fell into line with the others when a better grown culture was used.

The four strains of *C. pseudotuberculosis ovis* tested all caused death of both the test *and the control animals* in 24–48 hours. In two cases the protected animal lived a few hours longer than the unprotected but in the remaining two both animals died at practically the same time. Post-mortem all except one unprotected animal showed some degree of injection and oedema at the site of inoculation. The injection was always slight but in one unprotected and two protected animals the oedema was very marked. A varying degree of congestion was noted in various organs but macroscopic suprarenal changes when present at all were very slight compared with those usually found with *C. diphtheriae*. With two strains slight macroscopic changes were found in

the suprarenals of the test animal whilst those of the control showed none, but with the other two strains the position was reversed.

The pathogenicity of 65 of the diphtheroids in Series I was tested. The strains examined included all the members of Groups III, IV and V, 35 cane-sugar fermenters and 10 of the non-fermenting organisms in Group XI. In the second series all the strains in Group V and V*a*, two strains in Group III and one strain in each of the Groups II and XI were examined for virulence. The animals were kept under observation for a month or longer. Nine of the animals died 10 days or more after the inoculation. In some of these no obvious cause of death was found on post-mortem examination whilst others showed evidence of a "Gaertner" infection or pseudo-tuberculosis. The experiment was repeated in all nine cases and all the animals survived. Though it can be said that the inoculation of guinea-pigs with large doses of diphtheroids causes no mortality some of the animals did not gain weight at the normal rate; about one-third gained less than 30 gms. in four weeks Too much significance, however, cannot be attached to this observation as unfortunately no uninoculated controls were kept under the same conditions. The inoculations in Series I were made towards the end of August and in the first half of September there were rather severe morning frosts. It was thought that these might account in part at least not only for the failure to gain weight but also for some of the late deaths referred to above. The experiments at any rate show that the pathogenicity of the diphtheroids for the guinea-pig, if any, is on quite a different plane to that of *C. diphtheriae*.

Discussion of some of the groups.

Before concluding some points of interest in relation to one or two of the diphtheroid groups may be referred to. Though in discussing source, morphology and cultural characters it has been pointed out that no constant relationship was found between these and the biochemical reactions most of the groups contain strains which are indistinguishable from each other and may be regarded as examples of the same organism. This is a marked feature in Groups V, VI and VIII.

Group V.

The failure of dextrin as a reliable differential substance makes the position of the strains in this group of special interest, as apart from the absence of dextrin fermentation their biochemical reactions are those of *C. diphtheriae*. In Series I only one strain in this group was isolated from the nose and throat whereas out of a total of 13 in both series, nine are from nose and throat, two from the ear and one each from a whitlow and a genital swab. Eleven of the 13, all the nose, throat and ear strains appear to be representatives of the same organism. On agar these have all thick, white sticky growths which appear rather dry on the surface. On serum the growth is usually thicker and even in overnight cultures rather drier and rougher than that of *C. diphtheriae*.

Morphologically in pure culture on serum they are usually of moderate length or short, slender, beaded or striated and not infrequently very deeply stained. They can usually be distinguished from *C. diphtheriae* by the comparative uniformity both of the individual organisms and their arrangement. But in these strains, as in other members of the genus, variations in morphology have been observed from time to time. Though such variations have commonly lessened the resemblance to *C. diphtheriae*, the change at times has been towards a more diphtheria-like appearance. In the thinner parts of such smears the resemblance may be rather striking but even in those cases in the thicker parts the uniformity of arrangement usually gives a clue to the nature of the organism.

The genital strain only differs from those just described in that a yellow pigment is produced.

The distinguishing character of the remaining strain in the group isolated from a whitlow is that on both agar and serum the growth is very fine though the morphology is very similar to that of the other strains in the group. The fact that larger white variant colonies on one occasion appeared on an agar culture of this strain has possibly some bearing on its relation to the other members of the group especially as from time to time these thick growing strains have given thick and thin growths and even occasionally when picked on to agar from plates of Douglas medium growths in which small thin irregular colonies predominated, have been found. Considered by itself this group seems to be a very definite entity but Group III contains several strains in which the growth on agar and serum is indistinguishable from that just described as characteristic of Group V and having somewhat similar morphological characters. Yet these do not ferment galactose thus again emphasising the difficulty of completely correlating biochemical and other characters.

As already indicated, the special interest of the strains in Group V is whether they should be regarded as diphtheroids or as non-virulent strains of *C. diphtheriae*. In this connection it is of interest that all the four strains of this group in Series I had been regarded as diphtheroids on morphological or cultural grounds or both before their examination on the extended series of carbohydrates had shown their biochemical similarity to *C. diphtheriae* and before animal experiments had shown them to be non-virulent. The further study of these and the additional strains of the group in Series II confirms on the whole this opinion though it has shown that owing to occasional morphological and cultural variations there may be a temporary difficulty in placing them with certainty. It may be admitted that there is a certain temptation to build a theory of gradual loss of characters on the part of virulent strains but at present definite evidence in support of this is lacking. Should at any time, however, a virulent strain be found having the thick white sticky growth characteristic of Group V, the position of the strains in that group would at once have to be reconsidered, as owing to possible morphological variations and to the personal element which enters into the morpho-

logical diagnosis of doubtful strains of corynebacteria the growth on agar must be regarded as the chief point of distinction between this group and non-virulent strains which morphologically and culturally are quite indistinguishable from virulent *C. diphtheriae.*

Group Va.

Two strains both isolated from throat swabs within the last three months have been somewhat tentatively placed in this sub-group. Their biochemical characters differ only from those of the Group V strains in that the maltose reaction is definitely weaker. The latter almost invariably produce acid and clot in this carbohydrate whereas the reaction of those in the sub-group resembles more the incomplete and temporary reaction given by Groups I, II and IV in maltose. In one of the strains the fermentation of galactose is unusually slow. The fermentation reactions are almost the only point these two V*a* strains have in common with each other. The first has a thick yellow growth on agar and on both agar and serum the morphology is very similar to that of the Group V strains. The second strain has a thick moist sticky growth on agar and a very profuse moist growth on serum. Morphologically in pure culture on both media it resembles *C. hofmannii* very much more than *C. diphtheriae* though in the mixed culture from the throat it showed marked polar staining. Both strains like those in Group V are non-virulent.

Groups VI and VIII.

The strains in these two groups are only distinguished from each other by the fermentation of lactose by those in Group VI. As already mentioned (p. 244), this is a true fermentation of that carbohydrate to be distinguished from the temporary reaction given by *C. diphtheriae* and some diphtheroids. In morphology and the cultural characters studied the strains in the two groups are indistinguishable from each other. All have dry wrinkled growths on agar and thick rather dry growths on serum and all produce in some degree a yellow pigment (see p. 250). But the greatest interest of the strains is morphological. With the exception of one strain from the ear all were isolated from throat swabs and in smears from the primary mixed cultures the resemblance of individuals and small groups to *C. diphtheriae,* together with a certain pleomorphism at times gave rise to considerable difficulty in diagnosis. In the Diphtheria Monograph it is pointed out that the possibility of confusion with *C. diphtheriae* disappears in pure culture where they appear as short or more often very short slender rods staining somewhat irregularly or showing well-marked polar granules. Both the individuals themselves and their arrangement in relation to each other present an appearance of uniformity which is very different from that of *C. diphtheriae.* To exclude as far as might be the possibility that the diphtheria like forms seen in the primary cultures really represented *C. diphtheriae* which was subsequently overgrown by the second diphtheroid very exhaustive fishing of the plates was practised. This failed

to yield any evidence of *C. diphtheriae* and though in some cases a diphtheroid of another group was found (p. 248) in only one instance was it present in such small numbers that it would have been at all likely to have escaped observation in the ordinary examination of the plates. A further study of the morphology has also given support to the first opinion that the diphtheria-like forms seen in the primary cultures represent the diphtheroid and not *C. diphtheriae*; for from time to time though by no means constantly a morphology similar to that of the primary cultures has been found in pure culture. This was seen perhaps most frequently in young cultures (4, 6 and $7\frac{1}{2}$ hours growth) but has also appeared in older cultures. Even when present the diphtheria-like forms may be so few as to readily escape notice if not especially looked for and as a rule even in young cultures the predominant forms are those described as characteristic of these groups. On the other hand, occasionally in the same culture diphtheria-like morphology has predominated at one stage of growth and the group morphology at another.

Group XI.

It might be supposed that the non-fermenting organisms in Group XI were all representatives of *C. hofmannii*. Though strains of this organism are included other members of the group chiefly on morphological grounds could not be considered to be identical with it. These were often very short and slender, frequently somewhat solid with a rather indefinite median division; less commonly they showed well-marked polar staining. But with these as with other members of the genus the presence of polar granules was a somewhat variable character. The most interesting member of the group was one isolated from the ear of a child in an isolation hospital. It was of moderate length, slender, slightly curved, somewhat solid perhaps but often tapering off at one end and showing a polar granule at the other. The appearance suggested a young culture of *C. diphtheriae* but it ferments no carbohydrates and is non-virulent. It is probably an example of the *C. ceruminis* of other writers. Another strain in the group of nasal origin when first isolated was morphologically indistinguishable from *C. diphtheriae* though its morphology was of a different type from that just described. In some sub-cultures, however, the resemblance has been much less marked. Three strains in the group were distinguished from the others by the production of pigment. In two of these isolated from the nose the colour was a deep orange, in the third of genital origin it was a bright yellow.

The solitary strain in Group IX isolated from an oyster differs from all the other diphtheroids in the series in liquefying serum.

Mannite did not prove a very useful differential substance in the first series and was not used in the second. It was originally included because the great majority of previous workers who had used it in the examination of corynebacteria agreed that it was not acted upon by *C. diphtheriae*; whilst Douglas and his co-workers in their investigation of wound diphtheroids had

found a mannite fermenting group. In Series I mannite was only attacked by one strain (Group X). The reaction was slow, not appearing until 4th or 5th day and though definite was sometimes incomplete.

Differentiation of *C. diphtheriae* from the diphtheroids by means of fermentation reactions.

It will be noticed that Table II has been divided into three sections. Section A contains the groups which ferment some carbohydrates but not cane-sugar, B those fermenting cane-sugar and C the non-fermenters. This division of the groups is of interest because of its bearing on the possibility of separating virulent *C. diphtheriae* from all other members of the genus by means of fermentation tests. The existence of a number of strains, regarded as non-virulent *C. diphtheriae* which cannot be distinguished from *C. diphtheriae* either by biochemical reactions, morphology or other characters has already been alluded to. On the other hand, the fermentation of cane-sugar or the absence of fermentation of both glucose and cane-sugar have long been regarded as points of distinction between the diphtheroids and *C. diphtheriae.*

Of the 150 diphtheroids examined in the course of this work, 122 of which came from the ear, nose and throat, 71 per cent. from all sources or 74 per cent. if ear, nose and throat strains only are considered came into this category. Of the remainder those in Groups V and V*a* have the same fermentation reactions as *C. diphtheriae* but those in Groups III and IV 28 (18·6 per cent.) in all, of which 19 (15·5 per cent.) are from ear, nose and throat, do not ferment galactose and so can be distinguished from *C. diphtheriae* by that means. This seems to be a point of some practical importance particularly in laboratories having no facilities for animal experiments. Most of the strains in these two groups were isolated because of doubtful morphology in the primary mixed cultures. It is true that in *pure* culture many of them can be distinguished with comparative certainty from *C. diphtheriae* on morphological grounds. On the other hand, it is well known that corynebacteria are subject to considerable variations in morphology and that in doubtful cases the personal element enters largely into such diagnoses. In these circumstances any additional point of distinction would appear to be of considerable value.

The position of groups V and V*a* has already been very fully discussed (p. 254) but for reasons similar to those just advanced in favour of the use of galactose in the differentiation of Groups III and IV it is probably advisable in practice to regard all organisms fermenting glucose and galactose and not attacking cane-sugar as possible *C. diphtheriae* and to test their virulence even though the morphology and cultural characters of a few strains may justify an experienced bacteriologist in classifying them as diphtheroids.

SUMMARY AND CONCLUSIONS.

The fermentation reactions and some ancillary characters of 102 strains of virulent *C. diphtheriae*, 31 non-virulent strains and 150 diphtheroids have been studied.

With the possible exception of dextrin which proved to be an unsuitable substance for biological tests all the strains of virulent *C. diphtheriae* examined were found to have constant biochemical reactions, fermenting glucose, maltose and galactose and having no action on cane-sugar, lactose and mannite.

The strains classified as non-virulent *C. diphtheriae* had the same biochemical characters as the virulent strains and were indistinguishable from them either on morphological or cultural grounds.

The diphtheroids examined showed great diversity in their power of attacking the carbohydrates used, at least eleven biochemical groups being recognised.

By the use of three carbohydrates, glucose, galactose and cane-sugar 90 per cent. of the diphtheroids in this series could be distinguished from *C. diphtheriae* by their fermentation reactions. In the remaining 10 per cent. a clue as to the nature of the organism is often obtained from the character of the growth on agar.

Among the corynebacteria examined other than *C. diphtheriae* no constant relationship was found between source, biochemical, morphological and other characters.

Nevertheless in dealing with members of the genus in pure culture, the morphology, character of the growth on agar and fermentation reactions taken together give useful information as to whether the organism under consideration is *C. diphtheriae* (virulent or non-virulent) or a diphtheroid.

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