

A FURTHER ANALYSIS OF THE TYPES OF *CORYNEBACTERIUM DIPHTHERIAE* COMMON TO THE GLASGOW AREA

BY H. S. CARTER, M.D. (LEEDS), D.P.H.

From the Corporation of Glasgow Public Health Laboratory

IN a previous communication (Carter, 1933) an analysis of over 500 strains of *Corynebacterium diphtheriae* collected in the Glasgow district was described. Since then it has been possible to examine a much larger series upon which brief comment may be of interest. This series consists of 1614 strains derived from swabs sent for routine diagnosis by practitioners to the bacteriological laboratory of the Glasgow Public Health Authority, during the period May 1, 1934 to January 31, 1936, omitting only the Septembers of 1934 and 1935. It is consecutive and unselected, and probably represents with fair accuracy the picture of the disease in the Glasgow area, despite the fact that many cases of severe sore throat are sent into hospital without previous bacteriological diagnosis. This series reveals certain types of diphtheroid which did not appear in the first collection and which correspond to the Types IV and VI recently described by Wright and Christison (1935) as occurring in the Edinburgh area. The strains were isolated and typed by the use of McLeod's tellurite medium and the usual biochemical methods. The incidence of the various types was as follows:

1614 positive swabs					
<i>C. diphtheriae</i> <i>gravis</i>	Intermediate type	<i>C. diphtheriae</i> <i>mitis</i>	Type IV	Type VI	<i>Mitis</i> -like strains producing uniform turbidity in broth but fermenting starch
93	1052	389	10	66	4
(5.8 %)	(65.1 %)	(24.1 %)	(0.62 %)	(4.1 %)	(0.24 %)

These figures, except for the appearance of Types IV and VI, show no great variation from those of the earlier analysis of 510 strains which were divided into *gravis* 3.1 per cent., intermediate type 59.2 per cent., *mitis* 36.5 per cent., and aberrant *mitis*-like organisms 1.17 per cent. The relative incidence of the chief types is maintained. Nor does the percentage of the prevailing intermediate type differ greatly from the Edinburgh figure of 56.3 per cent. (Wright and Christison, 1935), or from the Dundee finding of 57 per cent. (Robinson and Marshall, 1934), such difference as there is being probably explained by the very much larger series examined over a longer period in Glasgow. *C. diphtheriae gravis* has been encountered rather more frequently than in 1933 and it occurs much more often than in the East of Scotland where

Wright and Christison (1935) found only four out of 469 strains (0·9 per cent.), and were unable to find any during the periods March–May 1934 and October 1934–April 1935 among the 303 strains examined. Evidently the intermediate type of *C. diphtheriae* has been for the past 3 or 4 years and still is the predominant type in Scotland and *C. diphtheriae gravis* has been of rare occurrence (Wright and Rankin, 1932; Carter, 1933; Robinson and Marshall, 1934; Murray, 1935; Wright and Christison, 1935). The *mitis* type has been found with diminishing frequency during the months of the present investigation.

CORYNEBACTERIUM DIPHTHERIAE GRAVIS

The type of illness and the end-results in eighty-nine infections with this type of organism (from May 1, 1934 to December 31, 1935) have been studied, and the following data emerge. The incidence of *gravis* strains varied from 2·35 per cent. in May 1934 to 13·2 per cent. in April 1935, an average of 4·9 per cent. per month during the period under investigation. The types of disease encountered and the incidence of complications were as below:

Mild cases 35	Moderately severe cases 31	Severe cases 15	Severe cases ending in death 8	
Paralysis 7	Albuminuria 5	Cardiac complications 17	Haemorrhagic 2	Hyperpyrexia 1

The incidence of complications is low, very much lower than in districts such as Leeds where for periods a very invasive type of *C. diphtheriae gravis* has been in the ascendant. All these cases received presumably adequate doses of antitoxin, from 40,000 to 120,000 units. Of the fatal cases, as far as can be ensured, four first received antitoxin on the second day of illness, three on the third day, and one on the fourth day. The fatality rate was 9 per cent.

A good deal of contentious matter has been written about colony variation, variation in the type of growth in broth, and stability of type in general in regard to the *gravis* strains (Wright and Rankin, 1932; Leete, McLeod and Morrison, 1933; Christison, 1933; Robinson and Marshall, 1934). It is to be noted that in their original paper Anderson and colleagues (1931) pictured a "less common" form of *gravis* colony without the architecture described as "daisy-head" formation. In Glasgow the colony appearances vary according to conditions of growth. Young packed colonies showing vigorous and profuse growth are usually smooth, flatly convex, circular, entire, iron-grey in colour, and of matt texture. These if planted out sparsely have invariably given in 48 hours "daisy-head" colonies showing little variation from the original conception. The growth has always been easily distinguishable from that of the *mitis* and intermediate types, though not so easily from that of Types IV and VI (see later).

Minor artificial variation in colony form obtained by varying conditions and expedients is of academic interest chiefly, though the realisation that this

may occur is of cautionary value in the initial typing of strains. Type diagnosis by sight on colony formation only is not infallible and consideration of the growth in broth and reaction in starch medium is often necessary for confirmation, the more so when *gravis* strains occur rarely, and the anomalous Types IV and VI are found, and where many cases of clinical severity yield intermediate forms; but there appears to be no sound evidence that *gravis* strains ever come up on the tellurite medium like *mitis* colonies in primary cultures, or *mitis* strains like *gravis* colonies, whatever transient modifications can be obtained by manœuvring with old broth cultures or by other experimental methods.

None of the ninety-three *gravis* strains grown in nutrient broth lysed human erythrocytes, but fourteen of them exercised some slight degree of haemolysis on rabbit's corpuscles. Towards the end of this work a few strains cultivated in 5 per cent. rabbit serum broth and tested against rabbit cells gave the impression of greater haemolysin production, but no actual comparative tests were made. Neither haemolysin production nor the rate of reversal of reaction in broth appears to be of more than slight confirmatory value in distinguishing the various types of *C. diphtheriae*, and indeed they appear to be unnecessary for that purpose, a conclusion which is upheld by the work of Wright and Christison (1935) and Robinson and Marshall (1934).

TYPES IV AND VI

Ten of the 1614 strains isolated corresponded to those named Type IV by Wright and Christison (1935) and sixty-six to their Type VI. Of the latter fifty-four have been gathered since June 1935. Before then this type was uncommon in Glasgow. Neither of these varieties was encountered in 1933. The clinical type of disease and the end-results of infections with these strains in nine cases of Type IV and fifty-nine of Type VI (covering the period up to December 31, 1935) are available.

Of Type IV cases, seven are to be recorded as mild and two as moderately severe. No complications were noted, progress was uneventful and there were no deaths.

Of Type VI cases, fifty-six were mild and three severe. Several of the mild cases were not obviously ill, and in most cases the throat signs were not very significant. In one household a familial infection of eight persons was discovered. Three only of these people were definitely ill, and one, a child of 2 years, died of acute bronchitis following tracheotomy for laryngeal diphtheria. One of the severer cases was described as severe faucial diphtheria with extensive exudate on both tonsils and uvula, and marked adenitis. The other severe case had a thick organised membrane and cervical adenitis. Both cases cleared rapidly and recovered without complications.

All the strains classified as Type IV were virulent for guinea-pigs and all those classified as Type VI were avirulent, most of the tests being by sub-

cutaneous inoculation of a saline suspension of 1/3 of a 16 hours' serum slant culture, and the remainder by the intradermal method.

Wright and Christison (1935) recognise that the authenticity of these anomalous types as true *C. diphtheriae* is arguable. All these Glasgow strains had the morphological, biochemical and cultural characteristics of *C. diphtheriae*. Morphologically they were large diphtheroids with abundant meta-chromatic granules. None of them fermented saccharose. They all developed good pellicles in broth indistinguishable from that of *C. diphtheriae gravis*. The colonial formation of both types on McLeod's tellurite medium was deceptive. In massed colonies the growth is lavish like that of *mitis* and *gravis* strains. The colonies are smooth, dome-shaped, circular and entire. The texture is semi-glossy and they are blacker than *mitis* and *gravis* colonies, there appearing to be more reduction of tellurite. Colonies planted sparsely grow very like *gravis* colonies, but are blacker and are not the typical daisy-heads of Anderson and colleagues' illustration (1931). There are radial striation, concentric grooving and often a small central papilla. The colonies are flatter than typical daisy-head *gravis* colonies. Type IV is indistinguishable from Type VI except by the virulence test.

Of Type IV, six strains and of Type VI, eight strains were tested for haemolytic properties against human and rabbit corpuscles with negligible results.

None of these anomalous *gravis*-like strains fermented starch, after either animal passage or prolonged growth in broth. Repeated subculture in starch medium did not cause fermentation of the polysaccharide.

In these series of cases due to Types IV and VI, three due to Type VI suffered severe illnesses and two due to Type IV moderately severe illnesses, and there was one death, although perhaps not directly due to diphtheria. A large number of these strains have been found and the organisms are present in the throats in large numbers. It certainly appears, then, that these strains must not be disregarded, as they apparently have more than academic interest epidemiologically and from public health and bacteriological aspects.

CORYNEBACTERIUM DIPHTHERIAE MITIS

Among the 510 Glasgow strains of *C. diphtheriae* examined in 1933 there were 186 or 36.5 per cent. of the *mitis* type. In the present longer series the percentage has dropped to 24.1, and the fall has taken place largely since December 1934. From May to December 1934 the percentage of this type was 31.1.

Most of the 389 cases infected with *mitis* strains were mild in clinical type, but fatalities were not entirely absent. In the first 239 cases from which *mitis* strains were isolated there were four deaths, or nearly 1.7 per cent., which is a very much smaller figure than was obtained in 1933 from a smaller number of cases and below the average given by McLeod (1935) in his analysis of 691 *mitis* infections, which is 3.3 per cent.

There was a small isolated outbreak of *mitis* diphtheria seen in a Training College. Seven persons had mild clinical diphtheria and three carried the organisms. All recovered without sign of complication.

Only four cases infected with aberrant forms colonially like *C. diphtheriae mitis*, producing turbidity in broth, no pellicle, but fermenting starch and glycogen, have been found, this figure comparing with six in the 510 cases of 1933. All four cases were mild and recovered without complications.

Although *mitis* type colonies may come up small (Anderson *et al.* 1933), especially if massed on the tellurite medium, the free edge of the growth is generally diagnostic, and there has been no difficulty here in distinguishing them from the other principal types. It is possible to mistake Types IV and VI in young culture for *mitis* strains, but the colonies of the former are not so glossy and are blacker, the reduction of the tellurite being more intense. Most *mitis* strains develop orange coloured pigment, seen well in old serum slant cultures.

THE INTERMEDIATE TYPE OF *CORYNEBACTERIUM DIPHTHERIAE*

This is still the predominating type in Glasgow as in Edinburgh and Dundee and probably may be considered the "epidemic type" in Scotland at present. It is responsible for the greater number of severe and fatal cases, and clinically infections with this type, if severe, may easily be regarded as *gravis* infections. Even so, and although *gravis* infections are somewhat rare, it does not cause the case mortality of the *gravis* type, although it is responsible, in Scotland at any rate, for most of the deaths from diphtheria. The case mortality, as far as can be ascertained in this Glasgow series, is approximately 7·8 per cent. The intermediate type of *C. diphtheriae* is of much more importance in Scotland, as the cause of by far the greater number of cases of diphtheria and also as a lethal agent, than the *gravis* type.

It is the easiest type to recognise on the tellurite medium, the fineness of young growth, looking like a grey-black powder scattered over the surface, being quite characteristic. Magnified, the colonies are as originally described Anderson *et al.* (1931), approximating to *C. diphtheriae mitis*, glossy with black centre, or, and this is the more frequent appearance of Glasgow strains much flatter and drier looking, semi-matt, with a tendency to radiating striation but still with a black centre raised, and a translucent margin. Even when planted sparsely, colonies of intermediate type never develop anything like the size of the other regular or anomalous types.

This type has never been found in these investigations to develop powers of polysaccharide fermentation, and its rate of reversal of reaction in broth has been found to be slow, but quite irregular.

No evidence of haemolytic properties of the intermediate strain has been obtained.

GENERAL SURVEY

Infections with *C. diphtheriae gravis* are rare in Glasgow (and indeed in Scotland), but when this is the infecting organism, two-thirds of the cases are severe. Nevertheless the incidence of complications seems to be lower among these sporadic cases than it is in epidemic areas, although the death rate is fairly high.

The intermediate type is the "grave" type for Scotland at present and causes most of the severe cases and most of the deaths. The case-mortality of diphtheria in Glasgow in 1933 was 3.8. In 1934 with many more cases it was 6.1, and this increase was due to infections with the intermediate type, for the increase in *C. diphtheriae gravis* infections, though significant as a percentage, was small in number, and *mitis* infections decreased in numbers and severity. Actually the percentage of *gravis* infections was greater in 1935 than in corresponding months of 1934, yet the case-mortality for 1935 was approximately 4.2 only.

The intermediate type caused a mortality of 15.7 per cent. in 231 cases recorded by Robinson and Marshall (1934), 13.5 per cent. in a small series of Leeds cases and 8.26 per cent. in McLeod's (1935) analysis of 1173 cases, and also the percentage incidence of complications shows little difference from that in *gravis* infections. Also, Anderson (1935) is able to record a case-mortality of only 3.8 per cent. in a consecutive series of 836 *gravis* infections.

There seems no doubt but that the intermediate type—probably the commonest type in Britain—must be accorded its place as an organism of great potential pathogenicity and had it been operative in Leeds in 1930–1, one cannot help feeling that it might have been given the leading role despite its lack of picturesque characteristics.

McLeod (1935) suggests as a subject for speculation:

"Whether the different forms of diphtheria described are permanently distinct as we believe typhoid para A and para B infections to be, with a tendency for different forms to predominate in different areas, e.g. paratyphoid A in warmer climates and the 'intermediate' type of diphtheria in northern areas."

This statement provokes the further suggestion, seeing that seven or more types of *C. diphtheriae* have now been described, of a comparison with the ever growing Salmonella group, and that a searching antigenic analysis might reveal untold complicating relationships.

SEROLOGY

No complete investigation of the serological relationships of the Glasgow strains has been made. An anti-serum to a typical *gravis* strain isolated from a very severe case which ultimately recovered was, however, made by intravenous inoculation of a rabbit, and a serum exhibiting a titre of 5000 to its homologous organism was obtained. The experimental methods and devices of Eagleton and Baxter (1923) to secure stable suspensions of the organisms

were used, and the results of agglutination experiments by Dreyer's methods were read after 4 hours' immersion in a water bath at 53° C. and confirmed after being allowed to stand on the bench overnight.

If specific agglutination to the full titre of the serum may be taken as good evidence of type, then there is, as in other places, more than one type of *gravis* strain in Glasgow. Of twenty strains tested, sixteen were agglutinated to full titre and four to various low titres, 50–500 only.

Cross-agglutinations of minor degrees were obtained by submitting various *mitis*, intermediate, and Type VI strains, and the anomalous *mitis*-like starch fermenters to the influence of the *gravis* serum. A few of the intermediate strains gave a stronger cross-agglutination up to 1000.

The *gravis* serum was absorbed by three suspensions of each type without altering the titre of the serum for its homologous organism, except in the case of two intermediate strains which had given a higher cross-agglutination titre. In these two cases the homologous titre was slightly reduced. Neither did the low-titre *gravis* strains, when allowed to react with the test *gravis* serum, materially reduce the titre of the latter.

There appears to be a definite difference serologically between the *gravis* and other types, though in some cases there may be group factors in common. It is likely that despite the different incidence of the original types in Scotland from that in other parts of the world, a large number of serological types is represented.

CHANGE OF FERMENTATIVE CHARACTERISTICS

Recently Murray (1935) has stated that:

“*C. diphtheriae* subcultured at 3-day intervals in broth containing 5 per cent. normal rabbit or normal guinea pig serum induced non-starch fermenting strains to ferment starch after 2 subcultures. . . .”

In other places in the same paper he says this phenomenon takes place after four subcultures, and also that it occurs if rabbit serum treated to 55° C. for 30 min. is used. It was not found possible to stabilise this acquired property.

To test these statements, nine *mitis* strains, six intermediate, and six Type VI strains were each inoculated into 8 c.c. of 5 per cent. fresh normal rabbit-serum broth (previously incubated for 48 hours to test sterility). These cultures were subcultured on to fresh tubes of the same medium every 3 days, using a 4 mm. platinum loop for transfer. Subcultures were also made on to starch medium ($\frac{1}{2}$ per cent. starch in Hiss' serum water, readily fermented by *C. diphtheriae gravis*) by the same method after one, two, three and four of the 3-day cultural periods in the serum broth. As was expected, although growth in the starch medium was profuse, there was no sign of fermentation, the medium remaining unchanged after a week's incubation.

However if the subinoculation on to starch medium were lavish, say 1 c.c. (carrying over 0.05 c.c. of rabbit serum), then starch fermentation took place in every case.

To each of several tubes of starch medium was added 1 c.c. of the serum broth and these were incubated for 48 hours, no change being visible at the end of this time. Now on inoculation of these tubes with non-starch fermenting strains of *C. diphtheriae* fermentation took place after incubation overnight, with bleaching of the indicator (phenol red) and coagulation of the medium.

The explanation seems to be, as was pointed out by Anderson *et al.* (1933), that the diastase of the serum, if there in sufficient quantity, hydrolyses the starch, and *C. diphtheriae* of any type will attack the products of the hydrolysis with acid formation and fermentation and coagulation of the starch medium.

The fact that the enzyme is not completely inactivated by heating the serum to 55° C. for 30 min. explains why heated serum may be substituted, and only when enough serum is carried over in subinoculation can any alleged change in the fermentative properties of non-starch fermenting strains be demonstrated. Starch paste is still acted upon by "diastase" which has been exposed to a temperature of 68° C. (Bayliss, 1914). The only explanation of Murray's results would seem to be that in some way a sufficient amount of serum was allowed to make contact with the starch to produce some hydrolysis, although it is true that the statement is made that ox, horse, human and sheep serum gave negative results, and that as little as 0.1 per cent. concentration in broth of anti-*gravis* or anti-*mitis* rabbit serum was sufficient to produce the change. It is perhaps significant that the change was so transitory that it did not survive one subculture on to solid medium.

SUMMARY

1. 1614 strains of *Corynebacterium diphtheriae* have been typed and analysed and some anomalous varieties described.
2. The various types have been considered in their relation to case severity.
3. Some comments are offered on the colonial and biochemical characteristics of the various types of *C. diphtheriae*
4. and on the serology of the strains found in Glasgow.
5. Two *gravis*-like strains Types IV and VI have been observed and correlated with clinical findings, and their cultural and biochemical characteristics described.
6. Attempt has been made to confirm alleged change in fermentative characteristics by growth in serum broth, with negative results.

ACKNOWLEDGMENT. I wish to express my thanks to Dr W. R. Wiseman, City Bacteriologist, Glasgow, for his help and advice during this investigation.

REFERENCES

- ANDERSON, J. S. (1935). *Brit. Med. J.* **1**, 1051.
- ANDERSON, J. S., COOPER, K. E., HAPPOLD, F. C. and McLEOD, J. W. (1933). *J. Path. and Bact.* **35**, 169; also *Lancet*, **i**, 293.
- ANDERSON, J. S., HAPPOLD, F. C., McLEOD, J. W. and THOMSON, J. G. (1931). *J. Path. and Bact.* **34**, 667.

- BAYLISS, W. M. (1914). *The Nature of Enzyme Action*. London.
- CARTER, H. S. (1933). *J. Hygiene*, **33**, 542.
- CHRISTISON, MAY H. (1933). *J. Path. and Bact.* **37**, 243.
- EAGLETON, A. J. and BAXTER, E. M. (1923). *J. Hygiene*, **22**, 107.
- LEETE, H. M., MCLEOD, J. W. and MORRISON, A. C. (1933). *Lancet*, ii, 1141.
- MCLEOD, J. W. (1935). *Univ. of Leeds Med. Soc. Mag.* ii, 26.
- MURRAY, J. F. (1935). *J. Path. and Bact.* **41**, 97 and 439; also *Brit. J. Exp. Path.* **16**, 532.
- ROBINSON, D. T. and MARSHALL, F. N. (1934). *J. Path. and Bact.* **38**, 73; also **39**, 551.
- WRIGHT, H. A. and CHRISTISON, M. H. (1935). *J. Path. and Bact.* **41**, 447.
- WRIGHT, H. A. and RANKIN, A. L. K. (1932). *Lancet*, ii, 884.

(MS. received for publication 13. III. 1936.—Ed.)