THE LIPOID ANTIGENS OF *C. DIPHTHERIAE* AND *C. HOFMANNII*

By L. HOYLE

(With 1 Figure in the Text)

Krah & Witebsky (1930) found that the sera of rabbits immunized with *C. diphtheriae* contained an antibody which would give complement fixation with alcoholic extracts of the organism. The sera also reacted with extracts of *C. hofmannii* and *Myco. tuberculosis*. From the results of absorption experiments they concluded that the lipoid antigens of *C. diphtheriae* and *C. hofmannii* were identical, and that two antigens were present, one of which was found also in tubercle bacilli.

This work was done before the description by McLeod and his colleagues (Anderson, Happold, McLeod & Thomson, 1931) of the *gravis*, *mitis*, and *intermedius* types of *C. diphtheriae*, and the object of the work described in this paper was to determine whether any differences existed between the lipoid antigens of the three diphtheria types. The term 'lipoid antigen' is used by the author to signify an alcohol-soluble haptene, the true chemical nature of which is unknown.

Rabbits were immunized by intravenous injections of formolized suspensions of *C. diphtheriae gravis*, *mitis*, and *intermedius*, and of *C. hofmannii*. Suspensions were approximately 5000 millions per c.c., and five doses ranging from 0·1 to 2·0 c.c. were given at 4-day intervals. Rabbits were bled 14 days after the last injection.

Alcoholic extracts were prepared from 48 hr. cultures on 5 % rabbit blood agar slopes, the bacteria being washed three times with saline, centrifuged out, and extracted with nine times their volume of absolute alcohol for 48 hr. For use in complement-fixation reactions the extracts were suitably diluted in saline by running the extract on to the surface of the saline and mixing by slowly rotating the tube. In most experiments the extracts were used diluted 1 to 40.

In the complement fixation reactions a constant dose of complement of 2½ M.H.D. was used. Each tube in the test contained: 0·2 c.c. of serum dilution (sera inactivated at 56° C. for 30 min.); 0·2 c.c. of complement (2½ M.H.D.); 0·2 c.c. of antigen dilution.

In some experiments the antigen was kept constant and a range of serum dilutions examined, while in other cases the reverse procedure was adopted. In all cases adequate serum and antigen controls were included. Fixation was for 1 hr. at 37° C., after which 0·4 c.c. of 3 % sensitized sheep cells (four doses of I.B.) were added to each tube. After a further incubation of 45 min. the tubes were placed in the refrigerator until the cells had settled, when readings were made.

It was found that the sera of rabbits immunized with *C. diphtheriae gravis*, *mitis*, and *intermedius*, and *C. hofmannii*, gave complement fixation with alcoholic extracts of the organisms, all four sera reacting with all four antigens. It was, however, noted that some of the sera reacted to higher titres with the homologous extract than with the heterologous

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extracts, suggesting that both specific and non-specific antibodies were present. A complete series of cross-absorption experiments was therefore undertaken, the sera being absorbed by suspending the absorbing organism in the serum to a concentration of some 5000 millions per c.c., incubating for 1 hr. at 37° C. and then centrifuging. From these experiments it was concluded that three distinct lipoid antigens, two specific and one non-specific, could be detected. The evidence on which this conclusion was based is given below.

(1) *C. hofmannii.* The original *C. hofmannii* serum reacted with all four antigens, though to very different titres. Absorption with *C. diphtheriae intermedius* rendered the serum specific for *C. hofmannii* (Table 1). Similar results were obtained by absorption with *C. diphtheriae gravis* and *mitis*. *C. hofmannii* must therefore contain at least two lipoid antigens, a specific one not present in any of the three diphtheria types and a non-specific or group antigen common to all four organisms. The specific antigen will be referred to as the *h* antigen, and the non-specific one as the G antigen.

Table 1. *Production of a specific* h *serum by absorption of* C. hofmannii *serum with* C. diphtheriae intermedius

Antigen 1:40	Serum dilution 1:2	1:4	1:8	1:16	1:32	1:64
	Original *C. hofmannii* serum					
gravis	+	0	0	+ +	+ + + +	+ + + +
mitis	+	0	+ +	+ + + +	+ + + +	+ + + +
intermedius	0	0	0	+ + + +	+ + + +	+ + + +
C. hofmannii	0	0	0	0	0	+ + +
	C. hofmannii serum absorbed with *C. diphtheriae intermedius*					
gravis	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
mitis	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
intermedius	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
C. hofmannii	0	0	0	0	+ + + +	+ + + +

Serum controls (1:2): original + + + +, absorbed + + + +. Antigen controls: *gravis* + + + +, *mitis* + + + +, *intermedius* + + + +, *C. hofmannii* + + + +. + + + + = complete haemolysis. +, + +, + + + = intermediate degrees of haemolysis. 0 = no haemolysis, i.e. complete fixation of complement.

(2) *C. diphtheriae mitis.* The original *mitis* serum reacted with all four antigens, but on absorption with *C. hofmannii* (Table 2) a specific serum was obtained, reacting only with *C. diphtheriae mitis*. The organism therefore contains in addition to the group antigen G a specific antigen which will be referred to as the *d* antigen. It was at first thought that the *d* antigen was found only in *C. diphtheria mitis*, but on absorption of the *mitis* serum with *C. diphtheriae gravis* the whole of the antibody was removed, and the same result followed absorption with *C. diphtheriae intermedius*. *Gravis* and *intermedius* must therefore contain the *d* antigen, though the amount present must be very small, since alcoholic extracts of the organisms did not react with the specific *d* serum obtained by absorbing the *mitis* serum with *C. hofmannii*.

(3) *C. diphtheriae gravis and intermedius.* The lipoid antigens of *C. diphtheriae gravis* and *intermedius* appeared to be identical. Thus it will be seen in Table 3 that absorption of the *gravis* serum with *intermedius* removed all the antibody, while absorption of the *intermedius* serum with *gravis* reduced the titre towards all the organisms though the whole of the antibody was not removed. It was found impossible to produce by any method of absorption a serum which would react with *gravis* and *intermedius* and not

Table 2. *Production of a specific* d *serum by absorption of* C. diphtheriae mitis *serum with* C. hofmannii

Antigen 1:40	Serum dilution 1:2	1:4	1:8	1:16	1:32	1:64
	Original *C. diphtheriae mitis* serum					
gravis	0	0	+ + + +	+ + + +	+ + + +	+ + + +
mitis	+	0	0	0	0	+
intermedius	0	0	+ + + +	+ + + +	+ + + +	+ + + +
C. hofmannii	+ +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
	Mitis serum absorbed with *C. hofmannii*					
gravis	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
mitis	0	0	0	0	+	+ + +
intermedius	+ +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
C. hofmannii	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +

Serum controls: original + + + +, absorbed + + + +. Antigen controls: *gravis* + + + +, *mitis* + + + +, *intermedius* + + + +, *C. hofmannii* + + + +. Significance of symbols as in Table 1.

react also with *C. hofmannii*, and it was concluded that in these two organisms the group antigen G is dominant.

The conclusions arrived at are illustrated diagrammatically in Fig. 1. All three diphtheria types contain the antigens *d* and G, but whereas in *C. diphtheriae mitis* the specific *d* antigen is dominant and the group antigen G present only in small amount, the reverse is the case in *C. diphtheriae gravis* and *intermedius*.

Table 3. *Identity of lipoid antigens of* C. diphtheriae gravis *and* intermedius

Antigen 1:40	Serum dilution 1:2	1:4	1:8	1:16	1:32	1:64
	Original *C. diphtheriae gravis* serum					
gravis	0	0	0	0	+ + + +	+ + + +
mitis	0	0	+ + +	+ + + +	+ + + +	+ + + +
intermedius	0	0	0	+ +	+ + + +	+ + + +
C. hofmannii	0	0	0	+ + + +	+ + + +	+ + + +
	Gravis serum absorbed with *intermedius*					
gravis	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
mitis	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
intermedius	+ + +	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
C. hofmannii	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
	Original *C. diphtheriae intermedius* serum					
gravis	0	0	0	0	+	+ + + +
mitis	0	0	+	+ + + +	+ + + +	+ + + +
intermedius	0	0	0	0	0	+
C. hofmannii	0	0	0	+ +	+ + + +	+ + + +
	Intermedius serum absorbed with *gravis*					
gravis	0	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
mitis	+ +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
intermedius	0	0	+	+ + + +	+ + + +	+ + + +
C. hofmannii	+	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +

Antigen controls all + + + +. Serum controls all + + + +. Significance of symbols as in Table 1.

C. hofmannii contains the group antigen G and also the specific antigen *h*, both being present in large amount.

No absorption experiment has given a result inconsistent with the antigenic structures indicated in Fig. 1.

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ANTIGEN ANALYSIS WITH SPECIFIC SERA

A consideration of Fig. 1 will show that it should be possible to produce specific sera against all three antigens, *d*, *h*, and G.

A specific *d* serum is readily produced by absorbing the *mitis* serum with *C. hofmannii*. A specific *h* serum may be produced by absorbing the *C. hofmannii* serum with any of the three diphtheria types, *intermedius* being best in practice.

A pure G serum can be produced by a *light* absorption of *gravis* or *intermedius* serum with *C. diphtheriae mitis*. This removes *d* antibody without greatly affecting the G titre.

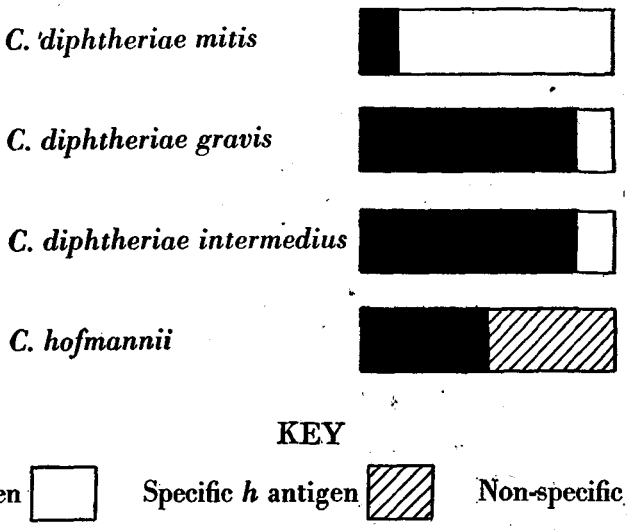

Fig. 1. Lipoid antigens of *C. diphtheriae* and *C. hofmannii*.

By means of specific sera prepared in this way an antigenic analysis of a number of strains of the various organisms was done. Alcoholic extracts of the organisms were made and falling dilutions tested for complement fixation with constant amounts of specific *d*, *h*, and G sera and with normal rabbit serum. Typical results are shown in Table 4. It will be seen that only the dominant antigens could be detected. Thus alcoholic extracts of *C. diphtheriae mitis* reacted only with *d* serum, while extracts of *C. diphtheriae gravis* and *intermedius* reacted only with G serum. Extracts of *C. hofmannii* reacted with both *h* and G sera. Alcoholic extracts of ten strains of *C. diphtheriae mitis* were tested; all reacted only with *d* serum. Extracts of eight *gravis* and six *intermedius* strains reacted only with G serum, while extracts of five strains of *C. hofmannii* reacted with *h* and G sera but not with *d* serum. A few extracts could not be tested because they proved to be anticomplementary.

PROPERTIES OF THE ANTIGENS

Certain differences in behaviour of the specific antigens *d* and *h* and the group antigen G were noted. The specific antigens appear to unite firmly and avidly with their corresponding antibodies; and these antibodies are easily removed from sera by absorption with quite small amounts of bacteria. By contrast, the group antibody G is more difficult to remove by absorption. A curious result of this difference in avidity of union was

encountered when the *intermedius* serum was lightly absorbed with *gravis*. All the antibody reacting with *C. diphtheriae mitis* was removed while the serum still reacted almost as well as before with *gravis*, *intermedius*, and *C. hofmannii*. This suggests that the *d* antibody is more readily absorbed than the G, so that although the amount of *d* antigen in *gravis* is small it is sufficient to remove all the *d* antibody, while the much larger amount of G antigen fails to remove the G antibody.

Table 4. *Antigen analysis with specific sera*

Serum	Antigen dilution 1:20	1:40	1:80	1:160
	C. diphtheriae gravis, no. 6 antigen			
Specific *h*	+	+ + +	+ + +	+ + + +
Specific *d*	+ + +	+ + + +	+ + + +	+ + + +
Group G	0	0	0	+
Normal rabbit	+ +	+ + + +	+ + + +	+ + + +
	C. diphtheriae mitis, no. 5 antigen			
Specific *h*	+	+ + +	+ + + +	+ + + +
Specific *d*	0	0	0	0
Group G	+	+.+	+ + +	+ + + +
Normal rabbit	+	+ + +	+ + + +	+ + + +
	C. diphtheriae intermedius, no. 5 antigen			
Specific *h*	+ + +	+ + +	+ + + +	+ + + +
Specific *d*	+ + + +	+ + + +	+ + + +	+ + + +
Group G	0	0	+	+ + +
Normal rabbit	+ + + +	+ + + +	+ + + +	+ + + +
	C. hofmannii, no. 4 antigen			
Specific *h*	0	0	0	+
Specific *d*	+ + +	+ + + +	+ + + +	+ + + +
Group G	0	0	0	+
Normal rabbit	+ + +	+ + + +	+ + + +	+ + + +

Serum controls all + + + +. Significance of symbols as in Table 1.

The group antigen G is probably not a single substance, but made up of a number of different lipoid antigens, the relative proportions of the components varying in different bacteria. Thus, while it is impossible to detect any qualitative difference between the antigens of *gravis* and *intermedius* by absorption methods, alcoholic extracts of these organisms always react more powerfully with the homologous than with the heterologous serum. The results suggest that quantitative differences in the amounts of the G components in the two organisms result in the production of sera with corresponding quantitative differences in antibody content, so that the sera tend to be better balanced against the homologous G antigen than against a heterologous G antigen in which the proportion of the components is different. This lack of balance when G antigen of one type reacts with G antibody of another type commonly results in the appearance of zone phenomena.

Antibody in human convalescent serum

A number of sera from human convalescents were tested for antibody content. Occasional sera, especially from *mitis* cases, reacted to high titres, but in general there was little antibody production, and most of the sera contained no detectable antibody. The positive sera proved more specific than the immune rabbit sera, those from *mitis* cases reacting only with alcoholic extracts of *C. diphtheriae mitis*. Some typical results are shown in Table 5. Sera from *gravis* and *intermedius* cases reacted with alcoholic extracts of *gravis*, *intermedius* and *C. hofmannii*, indicating the presence of G antibody, but in these cases it

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was almost always possible to determine the type of the infecting organism because the serum reacted more powerfully with the homologous extract than with the heterologous extracts. Serum 'Truscott' in Table 5 is a good example of this. The serum, derived from a case of infection with *C. diphtheriae intermedius*, reacted better with an extract of *C. diphtheriae intermedius* than with *gravis* and *C. hofmannii* extracts, with which latter extracts the zone phenomenon was shown. It was, however, found impossible to render

Table 5. *Antibody in human convalescent sera*

Antigen 1:30	Serum dilution 1:2	1:4	1:8	1:16	1:32	1:64	1:128
			Saunders (*mitis* convalescent) serum				
gravis	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
mitis	0	0	0	0	0	0	+
intermedius	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
C. hofmannii	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
			Maycock (*mitis* convalescent) serum				
gravis	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
mitis	0	0	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
intermedius	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
C. hofmannii	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
			Truscott (*intermedius* convalescent) serum				
gravis	+ +	+	+	+ + + +	+ + + +	+ + + +	+ + + +
mitis	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
intermedius	0	0	0	+	+ + + +	+ + + +	+ + + +
C. hofmannii	+ + + +	+	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
			Spencer (*gravis* convalescent) serum				
gravis	0	0	0	+ + + +	+ + + +	+ + + +	+ + + +
mitis	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
intermedius	0	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
C. hofmannii	+ +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +

Antigen controls all + + + +. Serum controls all + + + +. Significance of symbols as in Table 1.

this serum specific for *C. diphtheriae intermedius* by absorption with either *C. diphtheriae gravis* or *C. hofmannii*. One of the human sera examined gave an anomalous result. This was a serum from a *mitis* case which, unlike other sera from *mitis* cases, contained, in addition to *d* antibody, a large amount of G antibody. It was thought that this might be a case of cross-infection with *gravis* or *intermedius*, but no evidence was obtained which would support this view.

DISCUSSION

The experiments described above demonstrate that *C. diphtheriae* contains two lipoid antigens, a specific antigen *d* present in large amount in *C. diphtheriae mitis* and in small amount in *gravis* and *intermedius*, and a non-specific antigen G present in large amount in *gravis* and *intermedius* and in small amount in *mitis*. The G antigen is also present in *C. hofmannii* which contains, in addition, a specific antigen *h*.

These results differ considerably from those of Krah & Witebsky (1930), who concluded that the lipoid antigens of *C. diphtheriae* and *C. hofmannii* were identical. They detected only the G antigen, which they recognized to be composed of two components, one present in tubercle bacilli, and one absent from them. Their failure to detect the specific *d* antigen of *C. diphtheriae* was probably due to their working with *gravis* or *intermedius* strains. In the author's own work the presence of *d* antigen in *C. diphtheriae gravis* and *intermedius* could not be directly demonstrated by complement-fixation methods;

its presence was inferred from the fact that *d* antibody could be removed from a *mitis* antiserum by absorption with *gravis* or *intermedius*, and from the fact that *gravis* and *intermedius* sera reacted with alcoholic extracts of *C. diphtheriae mitis*. If, therefore, the diphtheria strains of Krah & Witebsky were *gravis* or *intermedius*, they would be unable to detect the *d* antigen. Their failure to detect the specific *h* antigen of *C. hofmannii* is, however, more surprising. An absorption of *C. hofmannii* serum with *C. diphtheriae* should have revealed the presence of the *h* antibody, but this absorption experiment is not described by Krah & Witebsky and was apparently not done. Witebsky & Krah (1930) noted that diphtheria antitoxic serum contained an antibody reacting with alcoholic extracts of *C. diphtheriae*, and that this antibody reacted also with *C. hofmannii*. It was therefore the G antibody, as might be expected, since antitoxic serum is made from the toxin of *C. diphtheriae intermedius*, in which organism the G antigen is dominant.

The author has used the term 'lipoid antigen' throughout this work in conformity with common usage to indicate an alcohol-soluble haptene, but it is of course possible that the various antigens owe their specificity to polysaccharide or other groups. In this connexion it is of interest to note that Wong & T'ung (1940) isolated a polysaccharide common to *C. diphtheriae gravis*, *mitis*, and *intermedius*, and not present in *C. hofmannii*, which organism contained a different polysaccharide.

Summary

1. Antisera prepared by immunizing rabbits with *C. diphtheriae gravis*, *mitis*, and *intermedius* and *C. hofmannii* contain antibodies which will give complement-fixation reactions with alcoholic extracts of the organisms.

2. By means of cross-absorption experiments three different lipoid antigens can be demonstrated: (*a*) a specific antigen *h* present only in *C. hofmannii*; (*b*) a specific antigen *d* characteristic of *C. diphtheriae mitis* but probably present also in small amount in *C. diphtheriae gravis* and *intermedius*; and (*c*) a non-specific or group antigen G present in large amount in *C. diphtheriae gravis* and *intermedius* and *C. hofmannii*, and in small amount in *C. diphtheriae mitis*; this G antigen is probably made up of a number of components.

3. Sera of human diphtheria convalescents may contain antibodies reacting with alcoholic extracts of *C. diphtheriae*. Sera from *mitis* cases usually react only with extracts of *C. diphtheriae mitis*, i.e. they contain only *d* antibody, while sera from *gravis* or *intermedius* cases usually contain only G antibody and react with extracts of *C. diphtheriae gravis*, *intermedius* and *C. hofmannii*, but not with extracts of *C. diphtheriae mitis*.

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REFERENCES

Anderson, J. S., Happold, F. C., McLeod, J. W. & Thomson, J. G. (1931). *J. Path. Bact.* **34**, 667.
Krah, E. & Witebsky, E. (1930). *Z. ImmunForsch.* **66**, 59.
Witebsky, E. & Krah, E. (1930). *Z. ImmunForsch.* **66**, 78.
Wong, S. C. & T'ung, T. (1940). *Chinese Med. J.* Suppl. **3**, 506.

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