The nature and properties of the haemagglutinin of *Clostridium welchii* and its relationship to the neuraminidase and other diffusible products of the organism.

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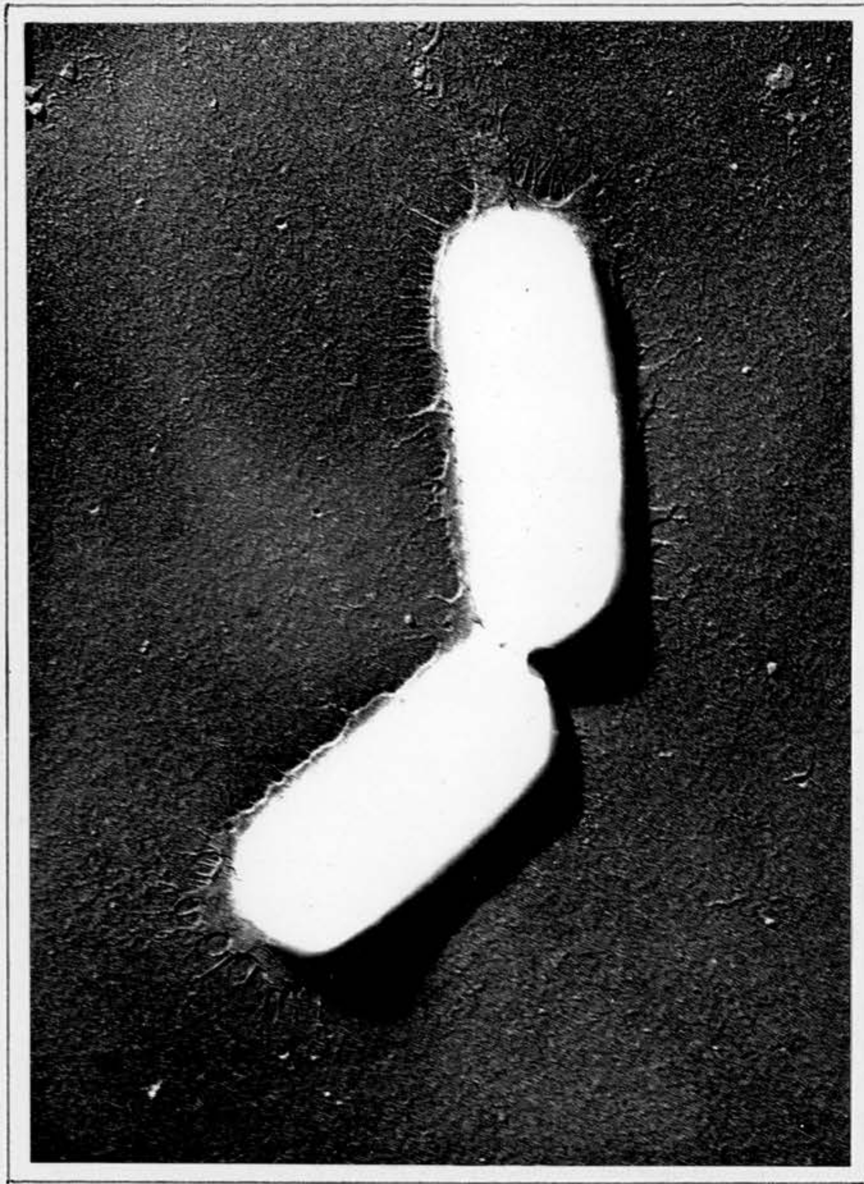

Clostridium welchii X 29,000
Electron micrograph of two cells fixed with formalin and shadowed with gold/palladium alloy.

PREFACE

Just as the investigation of the mechanism of myxovirus haemagglutination has enhanced our knowledge of systems involved in myxovirus - host cell interaction, so the further study of bacterial haemagglutinins may increase our understanding of bacterial activities at cell surfaces. The author here reviews the literature on direct bacterial haemagglutination with particular reference to conflicting reports regarding the haemagglutinin of *Clostridium welchii* and theories concerning its relationship to other products of the organism. In order to understand these complex inter-relationships it has been necessary to review our knowledge of such apparently unrelated topics as blood group substances, myxovirus haemagglutination, mucoprotein inhibitors and neuraminic acid complexes, neuraminidase, and the panagglutination (Thomsen-Friedenreich) phenomenon.

The haemagglutinin of *Cl. welchii* is characterised, its contested diffusibility studied and its rôle as a possible bacterial adhesive mechanism investigated. The unitarian hypothesis of Wickham (1956b), that the *Cl. welchii* haemagglutinin is identical with the neuraminidase and the enzyme of *Cl. welchii* that destroys

blood group-A substance (A-enzyme), is tested. The theory appears to be untenable, but Miss Wickham's approach to the problem is developed profitably.

The _Cl. welchii_ haemagglutinin is found to have much in common with the myxovirus haemagglutinin. Inhibitors of the _Cl. welchii_ haemagglutinin are detected in substances known to contain inhibitors of the myxovirus haemagglutinin. A hitherto unrecognised inhibitor of the _Cl. welchii_ haemagglutinin is demonstrated in cooked-meat broth and shown to be inactivated by _Cl. welchii_ cultures and by _Vibrio cholerae_ filtrate containing neuraminidase. The currently accepted method for the demonstration of neuraminidase activity, using myxovirus and red cells as indicator, is ultimately dismissed as unsuitable for the purposes of the present investigation in which interpretation of tests is frequently complicated by the co-existence of bacterial haemolysins and bacterial haemagglutinin. In addition, a temperature-independent system which blocks myxovirus receptors of red cells is detected in _Cl. welchii_ cultures. The limitations of tests employing red cells as indicator become evident and a search is made for a suitable substrate material with which to develop

a biochemical assay procedure for direct measurement of neuraminidase activity. A sensitive assay procedure employing hen egg-white as substrate is finally developed; strains of _Cl. welchii_ are then examined for neuraminidase production by a test which is independent of the red cell as indicator.

Tests of fractions obtained from individual cultures of _Cl. welchii_ afford further proof of the non-identity of the A-enzyme, the haemagglutinin and the neuraminidase, but it is evident that the haemagglutinin and the neuraminidase are closely related. The difficulties involved in a serological approach to the problem are now outlined.

Preliminary findings of an investigation of the panagglutination (Thomsen-Friedenreich) phenomenon are presented because this phenomenon may interfere in haemagglutination-inhibition tests incorporating serum. Provisional evidence of identity of the transforming principle with neuraminidase is found, but there is important evidence that another mechanism of transformation involving an adsorption process is also operative in certain _Cl. welchii_ cultures. This finding is discussed, separately from the main discussion, with reference to confusing reports in the recent

literature concerning the panagglutination phenomenon and neuraminidase activity.

The theory that the Cl. welchii haemagglutinin may be an altered form of its neuraminidase is submitted. The rôle of neuraminidase in the economy of Cl. welchii is discussed and its possible participation in mechanisms of pathogenicity is considered.

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The results of some of the work incorporated in this thesis have already been published.

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In a recent review of the literature on bacterial haemagglutination and haemolysis, Neter (1956) noted that the red blood cell has been of great use in microbiological studies during the past twenty years. Many of our laboratory tests which employ the red cell as a convenient indicator are still empirical, however, as we remain largely ignorant of the mechanisms which produce the visible phenomena we have learned to recognise. Studies of the actions of bacteria and bacterial products at the red cell surface are likely to yield useful information because, in addition to increasing our knowledge of the organism studied, they may shed light on the nature of the cell surface and on the mechanisms of its receptors. It is already evident that phenomena observed at the red cell surface are frequently similar to those occurring at the surfaces of epithelial and other cells so that theories based on such observations may have applications of wide biological interest. Thus, in the virological field, the initial discoveries of Hirst (1941) and McClelland and Hare (1941) concerning haemagglutination by the influenza virus led to intensive studies of the myxovirus haemagglutinins and their substrates and contributed much to our understanding of myxovirus-host cell interactions.

There are several known mechanisms whereby bacteria can bring about agglutination of red blood cells. Some involve sensitisation of the red cells to antisera by prior exposure to various agents including extracts of bacteria which either adhere to the red cell surface or alter it enzymically. Clumping of red cells by these methods is referred to by Neter as _indirect_ bacterial haemagglutination. _Direct_ bacterial haemagglutination, with which the work reported here is mainly concerned, is defined by Neter as the agglutination of red blood cells resulting from the action of bacteria or bacterial products and is independent of the presence of normal or immune serum. Direct bacterial haemagglutinins may be closely associated with the bacterial cell and are then termed "cell-bound" or _non-diffusible_. Alternatively, a soluble haemagglutinating substance may diffuse away from the bacterial cell into the surrounding medium and this is termed a _diffusible_ haemagglutinin.

Bacterial haemagglutination was noted at the beginning of this century by Kraus and Ludwig (1902) and Flexner (1902) who described haemagglutination caused by _Staphylococcus pyogenes_ and _Vibrio cholerae_ and by _Staphylococcus pyogenes_, _Pseudomonas pyocyanea_ and _Salmonella typhi_

respectively. Other early workers (Volk and Lipschutz, 1903; Kayser, 1903) observed bacterial haemagglutination; their observations were summarised by Pearce and Winne (1904) who found haemagglutinins in filtrates of cultures of several organisms including Salmonella typhi, S. cholerae-suis, Pseudomonas pyocyanea, Shigella dysenteriae, Staphylococcus pyogenes and Streptococcus pyogenes. Pearce and Winne thought that bacterial haemagglutinins were causally related in some cases to the development of thrombotic foci in the liver due to intravascular agglutination of red cells. At this early stage, these workers clearly confirmed the existence of diffusible bacterial haemagglutinins, although the essential differences between direct and indirect bacterial haemagglutination were not then recognised. A few years later, a non-diffusible haemagglutinin of Escherichia coli was described by Guyot (1908) and the history of the subsequent development of this work was recently outlined by Gillies (1959). Before proceeding to a detailed study of diffusible bacterial haemagglutinins it is advisable to review our knowledge of non-diffusible haemagglutinins, as considerable advances have been made since Neter (1956) expressed doubt at the concept of a cell-bound haemagglutinin and it is possible that the various mechanisms may be inter-related.

Non-diffusible bacterial haemagglutinins

The commonest type of haemagglutinating activity of *Esch. coli* was finally characterised by Duguid, Smith, Dempster and Edmunds (1955) who found that it was due to the presence on the bacteria of non-flagellar filamentous appendages which they called "fimbriae". These structures, first observed by Houwink and van Iterson (1950), occur in most strains of *Esch. coli*, *Aerobacter cloacae*, *Sh. flexneri*, *Salmonella*, *Klebsiella*, *Proteus* and *Serratia* (Duguid *et al.*, 1955; Constable, 1956; Duguid and Gillies, 1956, 1957, 1958)*. Fimbriate bacilli adhere to various substrates including red blood cells and cause haemagglutination by providing bacterial links between adjacent corpuscles. The haemagglutinin is an integral part of the bacterial cell and is therefore non-diffusible. It is most active in the cold at 4°C., haemagglutination being slightly weaker at 30 - 40°C. It is thermolabile and is inactivated within 1 hr at 80°C. (Gillies and Duguid, 1958). This type of haemagglutination is usually inhibited by small concentrations of D-mannose or α-methyl mannoside and is therefore said to be *mannose-sensitive*. Exceptions are found in *Proteus* (Gillies, 1959) and in some strains of *Klebsiella* and *Serratia* which possess

* see Plate 1.

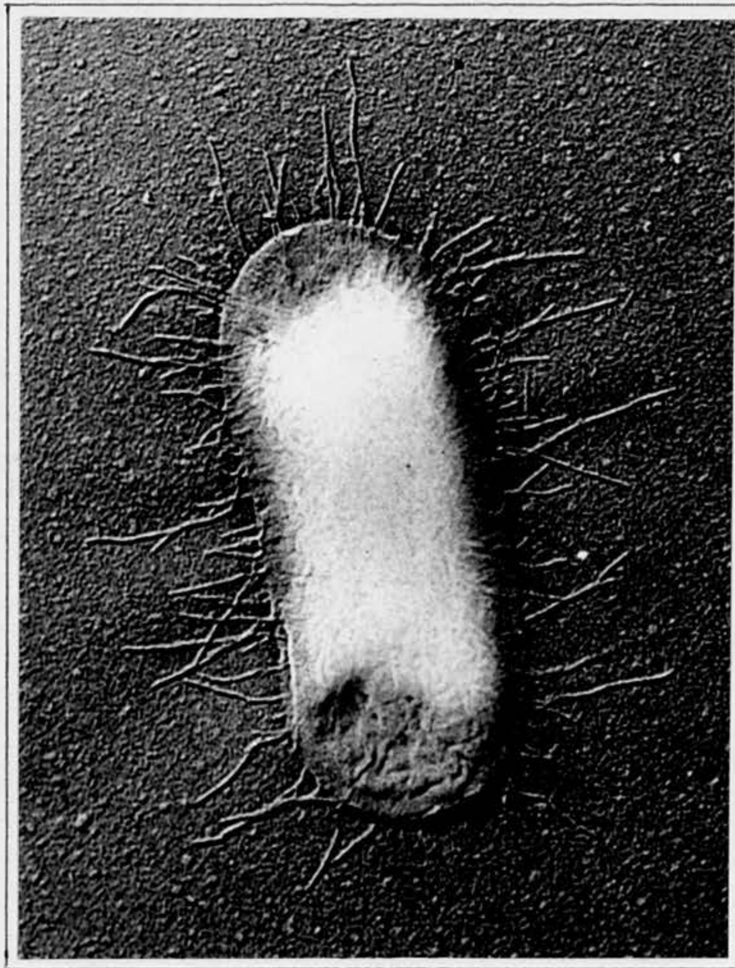


Plate 1. Electron micrograph showing typical fimbriae.
Escherichia coli X 40,000.
(Photograph by Dr. J.P. Duguid).

atypical fimbriae (Duguid, 1959), but, in general, fimbriate bacteria possess a non-diffusible, mannose-sensitive haemagglutinin or '*adhesin*' (Duguid and Collee, 1959).

Duguid *et al.* (1955) observed non-diffusible haemagglutinating activity in some strains of paracolon bacilli, *Bacterium freundii*, *Bact. cloacae*, *Alkaligenes faecalis*, *Salmonella typhi*, *S. paratyphi B*, *S. enteritidis*, *Shigella flexneri*, *Sh. sonnei*, *Pseudomonas pyocyanea*, *Proteus*, *Bacillus cereus*, *Corynebacterium diphtheriae*, *C. hofmanni*, *C. pyogenes*, *Pasteurella septica*, *P. pseudotuberculosis*, *Vibrio cholerae* and *Streptococcus pneumoniae*. Fimbriae were demonstrated in some of these but the authors correctly forecast that it was unlikely that the cell-bound haemagglutinating activities of such diverse species were in all cases due to the same kind of mechanism.

Constable (1956) stressed that red cells are convenient agglutinable particles and he attached no significance to the specificity of the fimbrial haemagglutination reaction as fimbriate organisms are known to agglutinate other cellular elements such as yeast cells. The fimbrial haemagglutinin certainly has affinity for apparently widely heterogeneous materials but it is not devoid of

specificity in that it is usually remarkably sensitive to mannose inhibition and also shows consistently varying degrees of affinity for red cells of different species. Thus it is possible that the haemagglutinin has affinity for a substance which is widely distributed in nature.

Fimbriate organisms are able to mutate readily to a non-fimbriate phase and may occur in the fimbriate phase only when there is a selective influence. Although there may be some association between fimbriae and fermentative capacity (Maccacaro and Angelotti, 1955; Duguid, personal communication) fimbriae are generally considered to be adhesive organs which, though not involved in all adhesive mechanisms of fimbriate organisms, provide a means of attachment to a wide range of cells. Many saprophytic organisms can adhere to mineral and organic solids by mechanisms unrelated to fimbriae and it is reasonable to presume that such organisms will benefit by growing in contact with surfaces where nutrients may accumulate by adsorption (ZoBell, 1943) or where the action of soluble lytic bacterial products may be economically concentrated (Duguid and Collee, 1959). Adhesive mechanisms may also be of use in retaining a saprophytic organism in a favourable environment, e.g. at the surface of

fluids by pellicle formation (Duguid and Gillies, 1957), or in contact with cells which may leak nutrient substances. Thus fimbriae may be of use in the nutrition of a commensal organism by enabling it to adhere to tissue cells *in vivo* or by maintaining its contact with shed epithelial cells. As fimbriae may be disadvantageous to an invasive pathogen and as it is evident that some invasive enterobacteria are permanently non-fimbriate, e.g. *Salmonella paratyphi A*, fimbriae are not thought to be involved in cell-surface attack and are most likely to be of advantage to an organism pursuing a commensal or saprophytic existence. Indeed, the optimal activity of the fimbrial haemagglutinin at temperatures well below 37°C. makes it unlikely that this adhesive mechanism subserves any pathogenic rôle in man unless an enzymatic mechanism co-exists which may rapidly liberate haemagglutinin inhibitor in the course of cell-surface attack.

Although we have evidence in the myxoviruses of adhesive mechanisms providing an initial means of access for enzymes involved in cell-surface decomposition, we have no proof that this type of mechanism occurs in fimbriate bacteria. However, there are some examples of apparently non-diffusible haemagglutinins which may exploit similar

mechanisms. Some strains of _Esch. coli_, including many that are haemolytic, possess a mannose-resistant haemagglutinin which appears to be non-diffusible but may be independent of fimbriation and is active for only a few red cell species (Duguid _et al._, 1955; Campbell, 1958). As this haemagglutinin is active in the cold at 4°C. but is spontaneously eluted from the red cells on warming them to 20 - 40°C., it has been likened to the haemagglutinin of the influenza virus combining with receptor substance at the red cell surface and, on warming, decomposing the receptor substance and disengaging itself (Duguid and Collee, 1959). It is doubtful if this analogy is now valid as it is evident that the process of agglutination and elution can be repeated frequently with the same test without gradual loss of agglutinability of the red cells involved. This would not occur if receptor destruction accounted for the elution of haemagglutinin on warming. It is nevertheless clear that more than one type of non-diffusible bacterial haemagglutinin exist and Duguid and Gillies (1957) recognised two types which differ in structure and activity:

(i) fimbrial haemagglutinins and

(ii) amorphous somatic haemagglutinins

Recent work by Campbell and Duguid (personal communication) has shown that non-haemagglutinating *Salmonella pullorum* and *S. gallinarum* are usually fimbriate. It is possible that the haemagglutinating activity of fimbriate organisms may depend on the accessibility of certain active groups on the fimbriae and therefore the possession of fimbriae alone would not necessarily confer haemagglutinating activity on an organism. If this is the case, it may ultimately be possible to dissociate the haemagglutinating factor from fimbriae and the distinction between diffusible and non-diffusible haemagglutinins would become less definite. Further work is necessary in this field.

Learned and Metcalf (1952) found that chemical extraction of *Corynebacterium diphtheriae* (gravis, Toronto strain) and *C. pseudodiphtheriticum* (hofmanni) yielded stable alcohol-soluble lipid antigens which agglutinated fowl red cells. Antisera prepared in rabbits against these lipid antigens gave positive complement-fixation and haemagglutination-inhibition reactions. Non-specific inhibitors of the haemagglutination reaction were associated with alpha- and beta-lipoprotein fractions of normal and immune sera, but specific inhibitor was associated with the

gamma globulin fractions of immune sera.

Several reported examples of haemagglutination due to non-diffusible haemagglutinins merit further investigation, e.g.

(a) Clostridium septicum haemagglutinin of Dafaalla and Soltys (1951) and

(b) Clostridium welchii haemagglutinin of Gürturk (1952); haemagglutinin of Dafaalla and Soltys (1953).

Dafaalla and Soltys (1951) described a haemagglutinin of Clostridium septicum which was produced in cooked-meat broth and blood agar cultures and readily agglutinated formalinised red cells of ox, sheep and fowl, being less active for red cells of horse, rabbit and guineapig. Haemagglutinin production in cooked-meat broth cultures was maximal after 24 - 48 hr and then fell with continued incubation until, after 10 days, there was virtually no activity. The presence of glucose in culture media appeared to reduce haemagglutinin yields. The haemagglutinin was mainly associated with the bacterial cells, spun supernatants of culture suspension being virtually inert. Dafaalla and Soltys used washed formalinised ox red cells and incorporated normal serum in their tests as it enhanced agglutination.

This makes it doubtful if these authors observed direct bacterial haemagglutination and it may be that indirect mechanisms, e.g. panagglutination (q.v.) were involved, although precipitated toxin preparations of _Cl. septicum_ did not sensitise red cells to antitoxic sera. The haemagglutinin was inactivated by exposure to 60°C. for 30 min. but resisted 24 hr exposures to 0.5 per cent. formalin, 0.5 per cent. phenol or 95 per cent. ethyl alcohol. Cultures on blood agar plates lost their cell-bound haemagglutinin after suspension in saline for 24 hr at pH 4.5 but retained haemagglutinin under similar circumstances at pH 7.0 and 9.0. The authors considered that this demonstrated instability of the haemagglutinin in acid although it may be that elution of cell-bound haemagglutinin occurred. The agglutinating factor was inhibited specifically by _Cl. septicum_ antibacterial and also antitoxic sera although it was thought to be non-diffusible and not associated with the _Cl. septicum_ toxins. It was not inhibited by antisera prepared against _Cl. chauvoei_, _Cl. welchii_ type D or _Cl. oedematiens_ type B, nor by normal rabbit serum.

The literature regarding haemagglutination due to _Cl. welchii_ is particularly controversial; this is discussed in detail below as it seems more likely that the _Cl. welchii_ haemagglutinin is a diffusible haemagglutinin.

Diffusible Bacterial Haemagglutinins

Certain bacteria produce haemagglutinating substances which diffuse into the surrounding medium and these diffusible haemagglutinins react with substrates at the red cell surface, altering the red cells so that they lose their normal mutually-repellent character. This may be the result of enzymic alteration of the red cell surfaces to an auto-adhesive state or, alternatively, the haemagglutinin may provide a multivalent particulate or macromolecular linkage between red cells following its adsorption. As diffusible haemagglutinins become independent of the bacterial cell they are unlikely to confer lasting adhesive properties on the bacteria which produce them. Like other haemagglutinins, they may be adsorbed by cells other than red cells, e.g. Clostridium botulinum haemagglutinin of Lamanna and Lowenthal (1951) which has affinity for the cells of many animal tissues (Lowenthal and Lamanna, 1953). The importance of observations concerning diffusible haemagglutinins in particular lies in the demonstrable affinities which they may reveal for specific substrates. These may provide clues of value in elucidating the interactions of bacterial products in the complex micro-environment at the

cell surface and in defining the possible mechanisms of cell-surface attack.

The first significant investigation of diffusible bacterial haemagglutinins was done by Keogh, North and Warburton (1947) who found that saline suspensions of _Bordetella pertussis_, _Bord. parapertussis_ and _Bord. bronchiseptica_, and the bacteria-free supernatants of these suspensions after centrifugation, agglutinated red cells of various species including man, mouse and fowl. Fisher (1948a) demonstrated that the haemagglutinating activity of liquid cultures of _Bord. pertussis_ increased sharply during the logarithmic phase of growth and as the cell counts of the cultures increased further their haemagglutinating activity decreased. As the pH of _Bord. pertussis_ cultures became more alkaline the young cells liberated haemagglutinin and, as autolysis continued, the haemagglutinating activity of the cultures decreased. Masry (1952) confirmed these observations and showed that, when grown on solid medium and also during the early stages of growth in liquid culture, the _Bord. pertussis_ haemagglutinin is associated with the bacterial cells but later diffuses into the medium. He extracted haemagglutinin from cultures grown on solid medium by suspension in

2*M*-sodium chloride or *M*-sodium acetate and he was able to precipitate the haemagglutinin from these extracts with methanol in the cold. Other workers (Fukumi, Shimazaki, Kobayashi and Uchida, 1953) found that methanol precipitation inactivated much of the haemagglutinin and they favoured precipitation with quarter-saturated ammonium sulphate. The haemagglutinin deteriorates rapidly, even at low temperatures. It is completely inactivated within a few minutes at 60°C., within 72 hr at 37°C., and within 4 days at room temperature. It was completely inactivated after 35 days at 4°C. and after 60 days at -10°C. Glycerol, 50 per cent., retarded the rate of deterioration (Masry, 1952). These findings were similar to those of Fisher (1948a, 1949) who found that the *Bord. pertussis* haemagglutinin was inactivated within 30 min. at 56°C. and could also be inactivated by agitation (Fisher, 1949). The haemagglutinating activity of the haemagglutinin occurs over a range of 4 - 50°C. (Masry, 1952). It is not dialysable through dialysing cellophan (Thiele, 1950). Masry found no correlation between capsulation and haemagglutinin production. *Bordetella pertussis* haemagglutinin is actively adsorbed to Seitz filters but only slightly adsorbed to

Gradocol collodion membrane filters (Masry, 1952). It is readily adsorbed on to aluminium phosphate at slightly acid pH (Warburton and Fisher, 1951) and is then more resistant to destruction by heat or agitation. The haemagglutinin, but not the toxin, is also readily adsorbed to fowl red cells or their stromata (Masry, 1952). Thiele thought that more than one haemagglutinin existed, as absorption of culture supernatants with one species of red cell did not remove all of the haemagglutinating activity for another species, but this could better be explained on the basis of availability of specific red cell receptors. <u>Bord. pertussis</u> is non-fimbriate and the haemagglutinin does not appear to confer enhanced adhesive properties on the strains which produce it (Sutherland, personal communication). The haemagglutinin is not spontaneously eluted from red cells after adsorption to them and it seems that it does not decompose its receptor as in haemagglutination by the myxoviruses. It can be inhibited by a lipid extracted from red cells and this may represent the actual red cell receptor substance for the haemagglutinin which does not depend for its activity on the presence of the influenza virus receptor at the red cell surface (Fisher, 1948b).

Antisera to the haemagglutinin were prepared by Masry (1952) by immunising rabbits with haemagglutinin in the form of extracts, bacterial suspensions or as haemagglutinin-saturated red cells. Active immunity to Bord. pertussis could not be conferred by injections of haemagglutinin, nor was it possible to confer passive immunity by injections of antisera to the haemagglutinin in experimental animals. Recently isolated virulent strains of Bord. pertussis usually produce good yields of haemagglutinin but some are non-haemagglutinating and, although prolonged subculture appeared to lead to parallel loss of virulence and haemagglutinin production in haemagglutinating strains, it is clear that the haemagglutinin is not the protective antigen concerned in the production of effective vaccines and it is distinct from the toxin (Pillemer, 1950; Standfast, 1951; Masry, 1952). Thiele (1950) thought that the haemagglutinating activity of Bord. pertussis cultures may be the result of some product of cellular metabolism which first appears as a transient characteristic of the young cells and is later utilised or destroyed in the later stages of growth. Sutherland and Wilkinson (personal communication) have recently shown that haemagglutinating strains

of Bord. pertussis, Bord. parapertussis and Bord. bronchiseptica produce a substance which is strongly haemagglutinating in the reduced state but loses its activity on exposure to oxygen. This inactivation is reversible. The substance is irreversibly inactivated by proteolytic enzymes. If all of the haemagglutinating activity of Bord. pertussis cultures is determined by their content of this substance, a ready explanation for the extreme lability of the Bord. pertussis haemagglutinin is available.

Davis, Pittman and Griffitts (1950) found that a diffusible haemagglutinin was produced by 27 of 28 strains of Haemophilus aegyptius. It was not produced by strains of H. influenzae, H. suis, H. para-influenzae and H. haemolyticus examined. The haemagglutinin is relatively stable, no loss occurring after 4 weeks at 37°C. It was not lost on subculture. The haemagglutinin had optimal activity over the pH range 6.6 to 7.6. It resisted 56°C. for 1 - 2 days but was destroyed within one minute in boiling water. Although it could not be washed off the bacterial cell, supernates and filtrates of spun cultures contained haemagglutinin. It was not inhibited by mucin but was specifically inhibited by antisera prepared by immunisation of rabbits which

were inoculated intravenously with formalinised suspensions of H. aegyptius cultures. The haemagglutinin was not eluted from the agglutinated red cells after 4 hr at 37°C. and red cells were not rendered inagglutinable by prior exposure to receptor-destroying enzyme. Thus the myxovirus receptors of red cells do not appear to be involved in this type of haemagglutination but the haemagglutinin is much more stable than that of Bord. pertussis. Smith (1954) examined 100 strains of Haemophilus spp. isolated from the conjunctiva and, of these, 33 produced haemagglutinin. He thought that haemagglutinating activity was frequently associated with pathogenicity but that the haemagglutinin itself was not the determining factor, as up to 50 per cent. of organisms isolated from clinical eye-infections were non-haemagglutinating. This appraisal of haemagglutinating activity in cultures of Haemophilus as an index of pathogenicity parallels current opinion on the correlation of haemagglutinin production with virulence in strains of Bord. pertussis.

Griffitts (1948) also found that saline suspensions of Shigella alkalescens, Bordetella bronchiseptica, Bord. pertussis, Vibrio cholerae and Streptococcus pyogenes are capable of clumping

human red cells. The haemagglutinin of *Sh. alkalescens* was studied in more detail. It can be separated from the bacterial cells by high-speed centrifugation after successive freezing and thawing of bacterial suspensions. It is labile, being destroyed within 1 min. at 93°C., and it is active with human, monkey and hog red cells. Although culture filtrates were non-haemagglutinating and disintegration of bacteria yielded only weakly haemagglutinating extracts, the haemagglutinin could be washed off the bacteria within 3 - 6 washings and appears to be essentially diffusible. The haemagglutinating activity was not dependent on temperature, occurring over a range of 5 - 56°C. Storage of a haemagglutinating culture at 5°C. for 6 weeks resulted in its subsequent loss of ability to produce haemagglutinin.

Collier and Jacoeb (1955) described a diffusible haemagglutinin of *Escherichia coli*, strain E 65, which could be washed off the bacterial cells after 1 - 2 washings. The haemagglutinin was active with fowl red cells but was not active with all human red cells. It was destroyed within 30 min. at 63°C. but was stable over the pH range 5.9 - 8.2 at room temperature. It was inactivated in 3 days by 0.5 per cent.

formalin. Collier, Sue Tsiew Wong and de Miranda (1955) demonstrated inhibition of this haemagglutinin to a greater or lesser degree with human urines irrespective of their albumin content. Inhibitors were also present in normal fowl and human sera and these were increased in their inhibitory activity by heating up to 100°C. Prolonged heating, e.g. boiling for 15 - 20 min., removed the inhibitor. Inhibitors were also found in extracts of spleen and brain of mice and guinea-pigs and in egg-white, but their inhibitory activities were not enhanced by heating. Collier and de Miranda (1955) showed that mannose, which is without effect on virus haemagglutination, had a remarkably powerful inhibitory effect on the haemagglutinin. As mannose was found by Duguid and Gillies (1957) to be a highly specific inhibitor of the non-diffusible fimbrial haemagglutinin, this observation of Collier and de Miranda needs further investigation. Collier, Tiggelman-van Krugten and Tjong a Hung (1955) found significant haemagglutinating activity in 53 of 105 strains of Esch. coli isolated from human faeces but only 2 of these produced a diffusible haemagglutinin. This could be absorbed by bacteria possessing non-diffusible haemagglutinating activity and by human, fowl and

guinea-pig red cells but not by sheep red cells.

Diffusible haemagglutinins are also produced by members of the Clostridia. Lamanna (1948) and Lamanna and Lowenthal (1951) described haemagglutinating activity associated with crystalline and amorphous preparations of the toxin of Clostridium botulinum, types A, B and E. Red cells of chicken, guinea-pig, rabbit, sheep and man were agglutinated. Particularly with sheep cells, the haemagglutinin was influenced by temperature, optimal activity occuring at low temperatures e.g. 2 - 4°C. The type A haemagglutinin was inhibited by type A Cl. botulinum antitoxin. Lamanna and Jensen (1952) showed that the haemagglutinin of Cl. botulinum type C was inhibited by antiserum to type C but not by antisera to type A or B.

Sterne (1954) found that the Cl. botulinum haemagglutinins lacked complete type-specificity, however, as type D haemagglutinin was inhibited by antisera to types C and D. Sterne also demonstrated partial inhibition of the Cl. botulinum haemagglutinin with high concentrations of a Cl. welchii and a Cl. oedematiens antiserum. The investigations of Lowenthal and Lamanna (1951) and Lamanna and Lowenthal (1951) indicated that the Cl. botulinum haemagglutinin differs from the

toxin which is more susceptible to inactivation by heat and formaldehyde but less stable at acid pH values of 3 and 2. The type A *Cl. botulinum* haemagglutinin was inactivated at 80°C. within 10 min. and lost much of its activity after 1 hour's exposure to 1 per cent. formalin at room temperature. These findings were apparently inconsistent with the reported homogeneity of the crystalline toxin demonstrated by diffusion, electrophoresis, ultracentrifugation and serological tests (Putnam, Lamanna and Sharp, 1948). However, it was clear that type B antitoxin neutralised the haemagglutinin but not the toxin of type A strains and type A antitoxin neutralised the haemagglutinin but not the toxin of type B strains. Moreover, type A antitoxin neutralised the haemagglutinin and toxin of type A culture supernatant disproportionately. Lowenthal and Lamanna (1953) then showed that the haemagglutinating and toxic properties of *Cl. botulinum* filtrates appeared to form a complex in acid solutions but became dissociated in alkaline solutions.

The *Cl. botulinum* haemagglutinin but not the toxin is adsorbed to red cells and numerous animal tissues other than red cells. Lowenthal and Lamanna (1953) considered that haemagglutination

by _Cl. botulinum_ filtrates is an adsorption phenomenon as the reaction has a negative temperature coefficient and does not appear to follow the law of simple multiple proportions. These workers thought that electrostatic attraction between the haemagglutinin molecules and the red cell surface was involved. They found that adsorption of haemagglutinin by sheep red cell stromata was greater in the presence of divalent ions than monovalent ions, cations being better than anions, and they noted that in this respect the reaction differs from the otherwise analogous mechanism proposed by Puck, Garen and Cline (1951) for myxovirus-host cell attachment. Lowenthal and Lamanna showed that no significant changes took place in the surface chemistry of the red cell following repeated adsorption and elution of _Cl. botulinum_ haemagglutinin and that the receptor sites are not the same as those involved in haemagglutination by the influenza virus; red cells treated with _Vibrio cholerae_ filtrate still agglutinated with _Cl. botulinum_ haemagglutinin, and red cells treated with _Cl. botulinum_ haemagglutinin, subsequently eluted, still agglutinated with influenza virus.

Gürtürk (1952) described a haemagglutinin of _Clostridium welchii_ active with fowl red cells.

His haemagglutination tests were done on a microscope slide. He stressed the importance of the cultural conditions and employed a modified Brewer's medium adjusted to pH 7.3 and omitting glucose. Haemagglutinating activity was maximal after 24 hr incubation and was associated with the spun bacterial deposit or "culture concentrate" resuspended after centrifugation in the residual 0.5 - 1 ml. fluid. Moreover, bacteria-free Seitz filtrates of haemagglutinating cultures possessed no haemagglutinin and the haemagglutinin appeared to be non-diffusible. Production of haemagglutinin was inhibited by the addition of glucose or calcium carbonate to the culture medium. Gürtürk prepared antisera to complete his range of available sera by inoculating rabbits subcutaneously with increasing doses of 24 hr liver broth cultures of Cl. welchii types E and F at 4 day intervals and he reported that useful sera were obtained about 3 days after the third injection. He noted that haemagglutination was inhibited by type-specific antiserum and that the stronger the haemagglutinin, the greater the amount of antiserum required to inhibit it. Antisera to different types of Cl. welchii inhibited heterologous haemagglutinin but Gürtürk claimed that type specificity was apparent at

higher dilutions. For example, he found that type C antiserum inhibited haemagglutination caused by types B, C, D, and E at a titre of 1 in 10 but only type C haemagglutinin was inhibited by the serum diluted to 1 in 20 and 1 in 40.

Dafaalla and Soltys (1953) found that the agglutinating factor in cultures of Cl. welchii, types A, B, C and D was associated with the bacterial cell and not the diffusible products of the organism. Washed cultures from agar were said to possess more haemagglutinin than those from fluid media, and the supernatant fluids from centrifuged broth cultures were almost inert. These workers grew Cl. welchii on 5 per cent. sheep blood agar plates for 48 hr and subsequently suspended them in physiological saline solution. The suspensions were treated with formalin "to inactivate the haemolysin" and were then titrated against fresh washed sheep red cells. Cl. welchii types B and D were more active than types A and C. Both antibacterial and antitoxic sera inhibited agglutination, but the latter were more effective. It was possible to distinguish type A from types B, C and D by haemagglutination-inhibition tests but no sharp differentiation could be made between the other types. Attempts to make the haemagglutination-inhibition test more specific

by prior absorption of antisera with suspensions of organisms were unsuccessful, nor was it found possible to remove anti-haemagglutinin by exposure of the antisera to red cells previously treated with the homologous strain. The authors concluded that the _Cl. welchii_ haemagglutinin is associated with the bacterial cell and is not sufficiently type-specific to be of use in typing _Cl. welchii_ strains.

Katitch (1954) investigated _Cl. welchii_ isolated from sheep and lambs dead of enterotoxaemia or lamb dysentery and found only 4 of 15 type B strains and 2 of 20 type C strains produced haemagglutinin. Five other laboratory strains of _Cl. welchii_, representing type A, D(2), E and F, produced no haemagglutinin. His methods of culture, haemagglutination and haemagglutination-inhibition testing, followed those of Gürtürk but Katitch could not classify his haemagglutinating strains reliably by haemagglutination-inhibition tests with type-specific antisera. Wickham (1956a) noted that whereas Gürtürk and Dafaalla and Soltys investigated laboratory-maintained strains, Katitch worked with strains which were predominantly freshly isolated and she showed that animal passage of a haemagglutinating _Cl. welchii_ strain resulted in selection of

non-haemagglutinating forms.

Wickham (1956a) studied haemagglutinin production in 14 stock strains of Cl. welchii, types A - D, and found that culture fluid freed from bacteria by centrifugation or Gradocol membrane filtration gave essentially the same haemagglutinin titre as whole culture of Cl. welchii. She therefore assumed, in contrast with Gürtürk (1952) and Dafaalla and Soltys (1953), that at least part of the haemagglutinin was diffusible and she was able (Wickham, 1955) to extract additional haemagglutinin from washed bacilli with 2 M sodium chloride, or M sodium chloride with 0.1 M sodium citrate. Wickham found that haemagglutinating Cl. welchii strains varied in their haemagglutinin production and that yields were dependent on the culture media used, cultures on sheep blood agar producing most haemagglutinin. Haemagglutinin yields were reduced when glucose, one per cent., was added to culture media and this was ascribed to acid production as a pH of 7.0 was necessary for optimal haemagglutinin production. Red cells of sheep, ox, pig, fowl and man (Group A) were agglutinated. Wickham's only consistent finding with regard to haemagglutinin production by various strains was that all haemagglutinin-

negative strains were freshly isolated. Moreover, she found that haemagglutinating strains became negative after guineapig passage. No difference in colony type or bacterial morphology, nor variations in production of spores or capsules could be correlated with differences in haemagglutinin production by the various strains. Cl. welchii α and θ antitoxins inhibited haemolysis but not haemagglutination, the potency of an antiserum to inhibit the haemagglutinin being related to the haemagglutinating capacity of the strain used in the production of the antiserum. The haemagglutinin of Cl. welchii appeared to be a group characteristic, unrelated to toxigenic types, as it was not inhibited by normal serum or by antisera prepared against other clostridia but could be inhibited by various antisera prepared against heterologous types of Cl. welchii.

Function of diffusible bacterial haemagglutinins

Although several diffusible bacterial haem-agglutinins are now known, there has been relatively little investigation into their possible functions. Just as the speculation of Burnet and his colleagues did much to accelerate further research on the myxovirus haemagglutinins, Wickham's attempt to define the relationship of the Cl. welchii haemagglutinin to other soluble products of the organism (Wickham, 1956b) invited further investigation in this important field.

Before reviewing Wickham's hypothesis, it is convenient to consider the knowledge of direct bacterial haemagglutinins summarised above and to postulate four reasonable theories regarding the possible functions of diffusible bacterial haemagglutinins:

(i) They may be produced initially as cell-bound non-diffusible haemagglutinins involved in adhesive mechanisms and, as a consequence of over-production, leakage or autolysis, become released from the bacterial cell.

(ii) They may occur as bacterial cell fragments, extracellular products of metabolism of the bacterial cell or diffusible enzymes which, by chance, have a molecular configuration resulting in the exposure of at least two reactive

groups; these may cross-link with accessible complementary groups on red cells.

(iii) They may be carrier molecules with multivalent affinity providing access to cell substrates for specific bacterial toxins or enzymes. They would act at a cellular level by agglutinating susceptible cells, or at a macromolecular level by causing aggregation and concentration of available substrate material.

(iv) They may represent enzymes similar to the enzyme of _Vibrio cholerae_ which destroys the myxovirus receptors of red cells (receptor-destroying enzyme or RDE). Enzymes of this type are believed to be concerned in cell-surface decomposition, attacking specific substances at the red cell surface, but their exact rôle remains in doubt.

More than one factor may be involved in some mechanisms of direct bacterial haemagglutination. The surface of the red cell may be altered on exposure to bacteria and bacterial products by enzymatic activity uncovering new receptor sites near the surface or by adsorption of substances at the red cell surface. While the concept of adsorption is embodied in the above theories, it is likely that if adsorption

or exposure of new molecules occurs, larger particles or aggregates may be required to provide links between these before haemagglutination can take place. Thus, Burnet, quoted by McCrea (1947), considered that the lecithinases of Cl. welchii and Cl. oedematiens have a distinct haemagglutinating effect on red cells which have been treated with V. cholerae RDE although they do not agglutinate normal red cells.

Haemagglutination is quite frequently noted in Cl. welchii haemolysin tests at dilutions just beyond the titre of the haemolysin. This phenomenon may be ascribed to mechanisms such as those envisaged above but it has not been satisfactorily explained. Oakley (personal communication) has observed haemagglutination in tests set up for theta toxin assay employing horse red cells and a reducing agent. Orlans and Jones (1958) described agglutination of red cells by filtrates of Cl. welchii cultures in haemolysin tests. Haemagglutination was not restricted to any one species of red cell but was found most commonly with horse red cells and the authors attributed this to the greater resistance of these cells to lysis. The haemagglutinating activity of cultures increased after longer growth periods (times unspecified) and did not appear to be

associated with any one type or toxin. Though sometimes suppressed by Cl. welchii type A antiserum, haemagglutination was more frequently enhanced in the presence of this antiserum. This suggests that the red cells may have been antigenically altered by the culture filtrate and reacted atypically with the antiserum (panagglutination, q.v.) but it seems that the lecithinase was not specifically involved.

Wickham's hypothesis

Wickham (1956b) considered that lecithinase, theta toxin and desoxyribonuclease did not participate in haemagglutination. She demonstrated that production of haemagglutinin by Cl. welchii was associated with three other properties of the organism collectively described by Chu (1948): (i) Destruction of blood group substance. Production by Cl. welchii of an enzyme apparently destroying blood group-A substance (A-enzyme) correlated with haemagglutinin production. (ii) Destruction of virus receptors of erythrocytes. Haemagglutinating strains of Cl. welchii produced an enzyme similar to the receptor-destroying enzyme (RDE) of Vibrio cholerae which destroys the myxovirus receptors in red cells. (iii) Panagglutination. Actively

haemagglutinating cultures of _Cl. welchii_ not only rendered human group-A erythrocytes more susceptible to group-A antiserum but rendered them susceptible also to group-B antiserum at the same high dilution, presumably due to enzyme action uncovering the non-group-specific T receptor near the red-cell surface (Thomsen, 1927; Friedenreich, 1928). There was a strong correlation between the production of these enzymes and of haemagglutinin in _Cl. welchii_ strains and Wickham noted that the RDE, the haemagglutinin and the A-enzyme were inhibited by the same _Cl. welchii_ antisera at approximately the same dilution. The haemagglutinin was also inhibited by a substance, assumed to be blood group-A substance, present in sterile cooked-meat broth. Wickham therefore suggested that all three activities of _Cl. welchii_, i.e. haemagglutination, receptor destruction and destruction of blood group-A substance, are due to the same enzyme and she implied that induction of panagglutinability of red cells is yet another manifestation of the same enzyme.

The purpose of the work to be reported has been to define more completely the properties of the _Cl. welchii_ haemagglutinin, including its contested diffusible character, to discover any

similarities it may have with other microbial haemagglutinins and to test Wickham's hypothesis which is outlined above. It is therefore necessary to review our knowledge concerning blood-group substances, receptor-destroying enzymes, haemagglutination by the myxoviruses, neuraminic acid and panagglutination.

Blood group substances

The ABO classification of human bloods (Landsteiner, 1900) defines four groups: A, B, AB and O. Anti-A or anti-B agglutinins occur naturally in the serum and specifically agglutinate red cells bearing the A or B factors respectively. The A and B factors, i.e. blood group substances A and B, are firmly bound to lipid components at the red cell surface and are there water insoluble. Many individuals also secrete their blood group substances which then occur in water-soluble form in their saliva, gastric juice, urine, etc. Blood group O persons do not possess either A or B substances but secrete a substance formerly called O substance because it neutralises various reagents which specifically agglutinate group O red cells. Blood group A and B individuals also secrete O substance and, as this is not therefore a specific character of blood group O persons, Morgan and Watkins (1948) re-named it H-substance. H-substance occurs in most red cells, tissue fluids and secretions and may be a precursor of A or B substances (Morgan, 1960). About 25 per cent. of all individuals do not produce A, B or H substances in water-soluble form and they are called "non-secretors".

Blood group substances were initially

obtained in impure form by ethanol extraction of red cells. Following the discovery of their presence in tissue fluids and secretions of secretors (Lehrs, 1930; Putkonen, 1930) they were obtained by various methods including alcoholic precipitation or phenolic extraction of secretions and tissue digests. Human ovarian cyst fluid (Morgan and van Heyningen, 1944) and meconium (Buchanan and Rapoport, 1951) were found to be good sources of blood group substance. There are various animal materials with blood group substance A, B and H specificity, glandular tissues and secretions such as gastric or intestinal mucosa and saliva of the pig, horse and cow, being particularly rich sources. It therefore follows that products incorporating these raw materials, e.g. pepsin, commercial peptones and gastric mucin, frequently contain significant amounts of material with blood group substance activity. This is important as the occurrence of blood group substances in bacteria including Esch.coli, and members of the Shigella, Salmonella and Proteus groups has been reported (Iseki, 1952; Iseki, Okada and Hayashi, 1952; Okada and Hayashi, 1953). Kabat (1956) has drawn attention to the observations of Stock (1939) and MacLennan, Mandl and Howes (1953) that contamination of

organisms with blood group substances derived from the culture media may occur. Springer (1955) avoided this pitfall by using synthetic culture media to confirm the occurrence in some bacteria of substance with A, B or H activity.

Structure and decomposition of blood group substances.

Blood group substances are mucopolysaccharides possessing a polysaccharide component firmly linked to an amino-acid-containing residue. They all appear to contain four sugars (L-fucose, D-galactose, D-glucosamine and D-galactosamine) and eleven or twelve amino acids. Mild acid hydrolysis produces gradual inactivation, but heating with very mild alkali, e.g. 0.05 N sodium carbonate, produces very rapid degradation. Watkins and Morgan (1955) showed that enzymic hydrolysis of blood group substances A, B and H can be specifically inhibited by a different sugar for each of the blood group substances. The sugar which inhibits the decomposition appears to be that which is normally liberated by the action of the particular blood group substance-destroying enzyme. Thus N-acetyl-galactosamine inhibits enzyme destroying A substance (A-enzyme), D-galactose inhibits B-enzyme and L-fucose

inhibits H-enzyme. These findings are consistent with the findings of other workers (Côté and Morgan, 1956; Kabat and Leskowitz, 1955, and Kabat, 1958) and suggest that these sugars are concerned in A, B and H specificity.

Enzymes capable of decomposing blood group substances have been obtained from sources as far apart as snail hepatopancreas (Freudenberg and Eichel, 1935) and fig latex and papaya fruit (Lawton, McLoughlin and Morgan, 1956). Enzymes splitting blood group substances are also produced by several micro-organisms. Much of the pioneer work in this field involved studies of Cl. welchii and this is reviewed in detail below but other organisms which are known to decompose blood group substances include: the myxobacterium of Morgan and Thaysen (1933) and Saccharobacterium ovale of Sickles and Shaw (1934), both investigated by Landsteiner and Chase (1935a,b); a Gram-negative coccus isolated from leaf-mould and investigated by Chase (1938); Cl. tertium (Iseki and Okada, 1951); Bacillus fulminans, a variety of B. cereus (Iseki and Tsunoda, 1952); Trichomonas foetus (Watkins, 1953) and Lactobacillus bifidus var. Penn (Springer, Rose and Gyorgy, 1954). Further work has been done to develop these findings by many workers, (e.g. Naylor and

Baer, 1959; Gilmore and Howe, 1959; and Morgan, 1960) and it is evident that this subject is of wide biological significance.

Schiff and Weiler (1935 a,b) found enzymes in normal faeces and saliva destroying blood group substances, and Schiff (1935) demonstrated enzymatic destruction of blood group substances A and B by a faecal strain of Cl. welchii. Nine of 16 strains of Cl. welchii, type A, and 2 other strains (types B and C) examined by Kostuch (1938) decomposed blood group substances and this activity could be inhibited by antisera prepared against the cultures. Kostuch failed to demonstrate that the decomposing factor was diffusible, culture filtrates being relatively inactive. He found no correlation between ability to decompose blood group substances and virulence in the strains he examined but noted that all of those strains decomposing blood group substances fermented glycerin. In 1939 Schiff described a diffusible enzyme of Cl. welchii which destroyed the blood group A substance, but not B substance, in saliva and peptone. This enzyme was unstable in acid at pH values below 6; it was oxygen-labile but could be protected or re-activated by ascorbic acid.

Morgan (1946) examined Cl. welchii, type A,

culture filtrates and confirmed the presence of enzymes which would rapidly inactivate the purified A, B and H substances. His results indicated that these activities were independent of the other known diffusible products of the organism. Stack and Morgan (1948) reported that separation of the enzymic activities of A- and B-enzymes had not been achieved. These workers in 1949 found culture filtrates from strains of _Cl. welchii_ representative of types A, C and D to contain about one tenth of the enzyme activity found for average type B filtrates. Tests on culture filtrates from a few strains of _Cl. histolyticum_, _B. anthracis_, _Staph. aureus_, _Ps. pyocyanea_ and _V. cholerae_ failed to reveal enzymes able to inactivate blood group substances. The organisms were grown on a peptone medium consisting of Evans peptone 3 per cent.; sodium β-glycerophosphate 2.5 per cent.; supplemented before inoculation with glucose 0.2 per cent. and thiolacetic acid 0.008 per cent.

"Without glucose, enzyme yields were generally one third less but without thiolacetate only one quarter of the enzyme production occurred. Glucose caused no stimulation of enzyme production unless thiolacetate was present. The addition of 0.01 - 0.20 per cent. of the test substrate

(mucoid purified from pig gastric mucin) caused no adaptive response. As group substances are present in the peptone used in the medium, the lack of adaptive response was confirmed by growing the same strain in the simplified medium described by Rogers (1945) with or without the addition of purified pig gastric mucin (A- and H-substances)."

Stack and Morgan (1949) confirmed Morgan's work (1946) in showing that the A- and B- enzymes are thermolabile in 1 hour at 56°C. in the absence of substrate, but they can be qualitatively differentiated from the H-enzyme by heating at 55°C. for 10 mins. owing to the relative stability of the H-enzyme in buffer (pH 7.0) at this temperature. "A preliminary incubation of the enzyme substrate mixture at 55°C. or 60°C. for 10 minutes prior to the usual 2-hour reaction period at 37°C. reveals that the A-enzyme is protected at 55°C. by its substrate, but not at 60°, while the H-enzyme is protected at 60°." Stack and Morgan also noted that if, after the usual enzyme-substrate incubation for 2 hours at 37°C., a further 1.0 mg. of substrate be added, repeated incubation results in complete inactivation of additional H-substance by the H-enzyme but the A-enzyme cannot inactivate the added A substances

completely, presumably because it loses the protection of its substrate towards the end of the first incubation period and is more labile. An associated depolymerase acting on an unidentified substrate in hog gastric mucin was found in the active filtrates and the inactivation by similar enzyme preparations of specific mucoids associated with the "Lewis" blood group characters, Le^a and Le^b (Mourant, 1946; Andresen, 1947), was also recorded (Grubb and Morgan, 1949). Some of the properties of the enzymes which have been investigated are summarised in table form below:

	A-enzyme	H-enzyme	De-polymerase
pH optimum	5.5	6.5	6.8
pH stability range	5 - 11	5 - 11	5.6 - 7.6
Temp. optimum (°C)	40 - 45°	40 - 45°	38°
Inactivation Temp. (10 min.) (°C)	55°	60°	56°
Growth optimum (days)	1	1	1

Stack and Morgan surmised that certain groupings on the enzymic complex which are specific for the A- or B-substrate (or the depolymerising activity) may become denatured, leaving other groups unaffected and still able to bring about the inactivation of the H serological character.

<u>Detection and assay of blood group A substance</u>

Undegraded A-substance inhibits the agglutination of group-A red cells by human blood group A antiserum and it also inhibits the haemolysis of sheep red cells by rabbit immune (anti-A) serum. The haemolysis-inhibition test is considered to measure the "Forssman" or heterophile component of the A agglutinogen and is accepted as measuring a different although closely related serological property from that determined by the isoagglutination-inhibition technique (Aminoff, Morgan and Watkins, 1950). Quantitative precipitin tests are employed for more precise determinations of blood group substances (see Kabat, 1956).

Extracts of seeds and other parts of many plants cause agglutination of red cells. The majority of these phytoagglutinins or <u>lectins</u> (Boyd and Shapleigh, 1954) agglutinate human red cells of all blood groups, but extracts from some plants are quite specific for red cells of certain groups, e.g. seed extracts of <u>Dolichos biflorus</u> and <u>Phaseolus lunatus</u> react with cells of groups A and AB but not with cells of groups B or O. Some preparations can be made virtually specific for sub-groups, and Boyd and Shapleigh suggest that the specificity of the lectins is a matter of varying degrees of affinity for different

antigens of one and the same molecule. Some of the lectins can also act as specific precipitins for blood group substances and, as the active precipitin can be purified and considerably concentrated by fractionation procedures, they are of great use in detailed studies of human blood groups. Cain (1957) has suggested that ingestion of lectins in food may have deleterious effects on the alimentary epithelium and considered that their neutralisation by the intestinal secretion of blood group substances is a protective mechanism of advantage to secretors.

Receptor-destroying enzyme

An enzyme of *V. cholerae* which destroys the influenza virus receptors of red cells was first characterised in 1946 by Burnet, McCrea and Stone and was called receptor-destroying enzyme (RDE). The production by *Cl. welchii* of a similar enzyme was first observed by McCrea (1947) who concluded that modification of red cell agglutinability by *Cl. welchii* toxins was caused by a non-dialysable, heat-labile factor which is extremely sensitive to pH changes. It was readily adsorbed on to red cells and thereafter eluted without significant loss of activity. In the range of *Cl. welchii* filtrates examined, he found a highly significant correlation between lecithinase and RDE activity but, as the two enzymes differed in heat-stability, optimum pH and adsorption characteristics, he concluded that the RDE of *Cl. welchii* is distinct from the lecithinase and the other known toxins and enzymes of *Cl. welchii*. The RDE was strongly inhibited by several *Cl. welchii* type A antisera in approximate proportion to their anti-lecithinase titres, but only slightly inhibited by purified alpha antitoxin. Moreover, RDE activity was found to be greatly accelerated in acid solution to pH 5.3 and was suppressed in

alkaline solution, whilst lecithinase has a broad optimum range between pH 5 - 8. The RDE was rapidly destroyed by heat in alkaline solution at 55 - 60°C. (e.g. within 10 mins. at 60°C. and pH 7.5) whereas lecithinase was not significantly affected by this treatment. McCrea pointed out that RDE can be absorbed from toxin solution without absorbing lecithinase. Fluoride, citrate, oxalate, and other ions suppressing calcium did not inhibit Cl. welchii RDE but actually facilitated its demonstration by minimising the haemolytic action of lecithinase. McCrea thought that although the RDE is not inhibited by ions which suppress calcium this is not entirely sound evidence for its non-identity with lecithinase which is calcium-dependent because the two systems are not strictly comparable in this respect. He considered that rapid adsorption of RDE to the red cell surface might nullify the effect of an inhibitor in the liquid phase. He had shown that, after an initial lag, the release of acid-soluble phosphate from red cell lipid by lecithinase proceeds normally in saline without added calcium, presumably because sufficient calcium is bound to the lipid. He maintained that lecithinase hydrolysis of lipids in emulsion is not entirely suppressed even in

the presence of citrate concentrations sufficient to prevent visible haemolysis in red cell systems. For these reasons, he and his colleagues had considered earlier that lecithinase produced receptor modification in the absence of calcium ions because its activity was retarded sufficiently to inhibit haemolysis without suppressing lipid hydrolysis, but he subsequently considered this improbable when it became evident that the lecithinase and RDE were separate enzymes. McCrea postulated concomitant production of these two enzymes by _Cl. welchii_ to explain the correlation of their anti-enzymes in most of the antitoxic sera he investigated. He also considered that there was competition between lecithinase and RDE and, although he doubted if a similar substrate was involved, he stated that the RDE effect was accelerated under conditions which selectively suppressed lecithinase. In contrast with Wickham's findings, he noted no significant alteration in the serum agglutination reactions of human red cells which had been incubated with an active _Cl. welchii_ strain until the red cells were no longer agglutinable by influenza virus.

McCrea found no essential differences between the characteristics of the RDE of _Cl. welchii_ and those of _V. cholerae_ RDE described by Stone (1947).

The two enzymes are similar in their relative thermostability in acid solution and in their thermolability at 55 - 60°C. in alkaline solution, in having acid pH optima and in being rapidly adsorbed to and quantitatively eluted from red cells. One point of difference is that the vibrio enzyme is inhibited by citrate and activated by calcium ions, neither effect being found with the *Cl. welchii* RDE. However, the action of the *Cl. welchii* enzyme is complicated by the presence of lecithinase, which as discussed earlier, may compete with the RDE at the cell surface; hence conditions apparently favourable to the RDE may in part be those least favourable to lecithinase. This would account for the observed optimum activity of *Cl. welchii* RDE at less than pH 6 and in the absence of calcium ions. The vibrio enzyme, on the other hand, is not produced concomitantly with lecithinase and is thought to indicate the truly optimum conditions for RDE activity.

Enzymes similar to the receptor-destroying enzymes of *V. cholerae* and *Cl. welchii* have been detected in cultures of several organisms including *Haemophilus influenzae* (Himmelweit, 1948); the pneumococcus (Chu, 1948; Ejby-Poulson, 1954a,b; Heimer and Meyer, 1956; Borecky, 1958 a,b;

Laurell and Bronnestam, 1959; Madoff, Eylar and Weinstein, 1960); diphtheroid and aerobic spore-forming organisms (Burnet, 1951); Actinomyces albus (Bergamini, 1956); Cl. tertium (Howe, MacLennan, Mandl and Kabat, 1957); Pseudomonas fluorescens, Ps. stuzeri, Ps. pyocyanea and Lactobacillus bifidus (Shilo, 1957); and alpha-streptococci and Pasteurella pseudotuberculosis (Laurell, 1959; Laurell and Brönnestam, 1959). All of these receptor-destroying enzymes have affinity for neuraminic acid complexes from which they cleave neuraminic acid and they are therefore termed neuraminidases (Gottschalk, 1957). Before the chemical decompositions produced by neuraminidases were understood, studies of these enzymes relied upon destruction of the influenza virus receptors of red cells as an indication of their activity. In order to understand the significance of these studies, it is necessary to outline our knowledge of the mechanism of haemagglutination by viruses of the influenza group (myxoviruses) and to summarise recent information concerning the important neuraminic acid complexes for which they have affinity.

Haemagglutination by the myxoviruses

The linkage of influenza virus with its host cells is established through at least two types of bonding at the same site (Ackerman, 1958). One bond involves a mucopolysaccharide receptor substance containing neuraminic acid and this has been extensively studied. Haemagglutination by the myxoviruses is brought about by adsorption of virus particles to red cells at receptor sites containing N-acetyl neuraminic acid. The myxoviruses possess neuraminidase activity and release neuraminic acid derivates from red cells and from mucoprotein inhibitors of virus haemagglutination which contain neuraminic acid and compete for the virus particle. As a result of their neuraminidase activity, the myxoviruses are normally eluted some time after adsorption at the red-cell surface when the virus receptors on the red cells are destroyed; the virus can then attack fresh receptor sites and the red cells are eventually rendered inagglutinable, i.e. stripped of receptors, even for fresh virus subsequently added. Briody (1948) and other workers found that influenza virus which had been heated at 55°C. for 30 min. retained its haemagglutinating activity but lost its ability to be eluted after adsorption to the red cell. In addition, heat-

inactivated virus cannot destroy mucoprotein inhibitors of virus haemagglutination. Its haemagglutinating activity is therefore particularly sensitive to inhibition by these substances and the heated virus is said to be in the "indicator state". Work with heat-inactivated (indicator) virus and unheated (active) virus revealed that human serum may contain three inhibitors of virus haemagglutination:

(i) antibody, present in immune serum;

(ii) non-specific inhibitor which inhibits haemagglutination by active virus;

(iii) Francis inhibitor, described by Francis (1947), which inhibits haemagglutination by indicator virus.

The non-specific inhibitor content of a serum is considerably lower than its Francis inhibitor content but both of these mucoid inhibitors are destroyed by exposure to *V. cholerae* culture filtrates containing RDE (Anderson, 1948). Gottschalk (1960) observed that, although all mucoproteins containing neuraminic acid appear to be susceptible to neuraminidase, only some of them inhibit haemagglutination by influenza virus. He suggested that an inhibitory mucoprotein must not only provide substrate material for neuraminidase in order to inhibit haemagglutination but

must also compete successfully with the red cell receptor for the virus particle.

Burnet, McCrea and Stone (1947) showed that the myxoviruses can be arranged in a gradient according to their power to destroy the red cell receptors available to themselves and to all other myxoviruses lower on the gradient, e.g. the influenza viruses destroy receptors more actively than the virus of Newcastle disease (NDV) which is higher on the gradient than the weakly active virus of mumps. A gradient of accessibility of receptors at the red cell surface was therefore postulated. A similar but not identical gradient of ability to destroy various inhibitory mucins was demonstrated (Stone, 1949; Hirst, 1950). To some extent, the order of the viruses on the inhibitor destruction gradient depends upon the mucin substrate on which the viruses are tested. Moreover, tests of destruction of receptors with different viruses showed that all strains could remove all of the receptors if given sufficient time. Thus, Hirst favoured the idea of a mosaic, at the red cell surface, of receptor sites for which the viruses have different affinities.
The available evidence also suggests that the haemagglutinating and receptor-destroying (neuraminidase) activities of the myxovirus particle represent closely related but separate

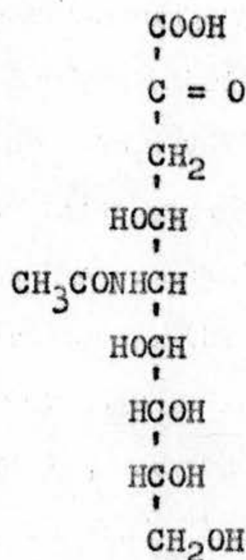
steps in its attack on cell surface neuraminic acid substrates.

Neuraminic acid

Neuraminic acid does not occur naturally. It is the parent compound of acylated derivatives which are widely distributed and, having been first obtained from various animal submaxillary mucins, are known as the sialic acids. Five sialic acids have been isolated. These differ in the nature or position of their attached acyl groups and were termed ovine, porcine, equine and bovine I and II, but these substances are not confined to the animal species from which they were first isolated. Different sialic acids occur together in animal tissues and sera. The sialic acids occur as mucoproteins in blood, mucins and milk and as mucolipids in certain lipopolysaccharides. Sialic acid has been detected in purified blood group substance but its presence may be due to contamination. Neuraminic acid derivates have been isolated from certain bacteria, e.g. *Escherichia coli* (Barry and Goebel, 1957); *Neisseria meningitidis* (Watson, Marinetti and Scherp, 1958).

Neuraminic acid (prehemataminic acid) may be regarded as an aldol condensation product of pyruvic acid with either D-glucosamine or D-mannosamine, and the most likely configuration

for N-acetyl neuraminic acid, according to Comb and Roseman (1960), is:



Human sialic acid appears to be solely of this type (synonyms: ovine sialic acid, gynaminic acid, lactaminic acid, serolactaminic acid). Details of the sources and methods of isolation of sialic acids with data on their characterisation and identification have been summarised by various authors including Blix, Lindberg, Odin and Werner (1956); Gottschalk (1960); Whitehouse and Zilliken (1960) and Comb and Roseman (1960). Determination by colorimetric methods is widely practised because the sialic acids exhibit characteristic colour reactions in acid media with several reagents such as:

(1) p-dimethylaminobenzaldehyde (direct Ehrlich test,

(2) orcinol (Bial's reagent),

(3) diphenylamine (Dische's reagent),

(4) tryptophan in perchloric acid (Siebert, Pfaff and Siebert, 1948; Werner and Odin, 1952), and

(5) thiobarbituric acid after oxidation with sodium periodate (Warren, 1959).

Assay of neuraminidase

The chemical assay of neuraminidase (RDE) depends upon the demonstration of its enzymatic liberation of sialic acid from sialic-mucoprotein complexes. Aminoff (1959) pointed out that those methods which involve heating with an acidic reagent liberate glycosidically bound sialic acid from mucoproteins and therefore do not distinguish between free and bound sialic acid. It is therefore necessary after incubating the enzyme-substrate mixture to precipitate any residual protein-bound sialic acid and remove it before assaying liberated sialic acid by a method which would otherwise cause acid hydrolysis of the residual bound substance. While no colorimetric method of sialic acid assay is entirely specific, the thiobarbituric acid assay developed by Warren is claimed to be considerably more specific and is certainly more

sensitive than the others mentioned. It is of great use in neuraminidase studies as it measures only unbound (liberated or "free") sialic acid. Bound sialic acid can be measured by Warren's method if it is initially liberated by mild acid hydrolysis, e.g. treatment with 0.1N sulphuric acid for 1 hour at 80°C. This is successful provided that the sialic acid is bound by labile (e.g. glycosidic) linkages, as in mucolipids and many mucoproteins. Whitehouse and Zilliken pointed out however that the ester or peptide bonds which hold the neuraminic acid residues in certain bacterial neuraminopeptides are not labile. Attempts to hydrolyse these usually result in decomposition of the neuraminic acid present. Thus free sialic acid can be sensitively identified but it may be necessary to employ less sensitive methods to detect the bound substance in bacteria.

A further possible complication in bacterial neuraminidase studies is that enzymes may be produced which can destroy sialic acid (Heimer and Meyer, 1956). Such an enzyme is produced by Cl. welchii (Popenoe and Drew, 1957) and has been characterised as an aldolase (Comb and Roseman, 1958). It is clear that an assay of neuraminidase activity based on the liberation of free

sialic acid from a mucoprotein substrate by a culture filtrate must be performed under conditions unfavourable to simultaneous destruction of the liberated sialic acid by another enzyme.

Panagglutination

Many organisms produce enzymes or "transforming principles" which alter or transform the antigenic character of human red cells, rendering them panagglutinable, i.e. agglutinable by all adult sera regardless of ABO specificity. This phenomenon was first studied by Heubener (1926), Thomsen (1927) and Friedenreich (1928) and was attributed to the unmasking of an antigen near the red cell surface, the T antigen, which can then react with T-agglutinins normally present in adult mammalian sera but absent from cord blood and blood of young infants (Lind and McArthur, 1947). The T-agglutinin content of animal serum can be increased by immunisation with red cells treated so that their T antigens are exposed (Burnet and Anderson, 1947). Red cells of animals are also susceptible to panagglutination and, in the presence of complement, T antibodies react with transformed guinea-pig red cells to produce haemolysis after initial haemagglutination (Stone, 1947). Transfusion of transformed red cells causes shock and a syndrome similar to paroxysmal haemoglobinuria in guinea-pigs. Transformation of guinea-pig red cells by pneumococci *in vivo* was effected by Ejby-Poulsen (1954 a,b). Injection of pneumococcal culture

filtrates into guinea-pigs with high T-agglutinin serum levels produced a haemolytic anaemia.

The characteristics of the factor concerned in exposing the T antigens of red cells were summarised by Neter (1956). It is thermolabile and filterable. It is most active in transforming red cells at 15 - 20°C. It is rapidly adsorbed to and subsequently eluted from the red cell surface and it is not re-adsorbed by transformed red cells. It does not itself cause haemagglutination, nor do T-antibodies agglutinate the bacteria producing transforming principle. T-agglutinins are absorbed by transformed red cells but not by normal red cells.

In addition to the diphtheroid organisms "M" and "J" originally studied by Friedenreich, organisms known to produce transforming principles include _V. cholerae_ (Stone, 1947); _V. proteus_, _Cl. welchii_ and the _pneumococcus_ (Chu, 1948); _Cl. tertium_ (Howe _et al._, 1957) and _Trichomonas foetus_ (Watkins and Morgan, 1954). Most of these organisms are known to produce receptor-destroying enzymes. Moreover, treatment of red cells with the myxoviruses renders the red cells panagglutinable (Burnet, McCrea and Stone, 1946). Thus this type of change is thought to be "almost certainly due to the action of receptor-destroying

enzyme" (Stewart, Steele and Martin, 1959).

Induction of panagglutinability cannot, however, be considered a reliable index of RDE activity for several reasons. It has not been proved that RDE and the agent inducing panagglutinability are identical. Chu (1948) demonstrated induction of panagglutinability by cultures of several organisms which did not simultaneously produce receptor destruction. The optimal activity of the transforming factor at 15 - 20°C. differs from that of the RDE which occurs at a higher temperature. The available evidence indicates, nevertheless, that the two activities may be different manifestations of the same type of enzyme; receptor-destruction may involve a greater decomposition of substrate than the modification necessary for induction of panagglutinability.

Much work remains to be done to correlate observations of several workers regarding panagglutination produced by many different organisms. Interpretation of results in this field is difficult because there are other mechanisms of inducing panagglutination independent of the T-agglutinin:

(a) Von Magnus (1936) produced T-independent panagglutinability of red cells by treatment with

a strain of actinomyces.

(b) Hirst (1948) and Burnet (personal communication to Briody, 1948) observed that periodate ions rendered red cells panagglutinable; Stewart (1949) and Moskowitz and Treffers (1950) showed that this phenomenon was distinct from that producing panagglutination of red cells treated by V. cholerae culture filtrates.

(c) Moskowitz and Treffers found, in addition, that Staph. aureus culture filtrates contained a factor causing yet another type of panagglutination, independent of the periodate and T mechanisms, and they concluded that there is more than one panagglutinin in serum.

(d) Stewart, Steele and Martin (1959) then showed that panagglutination could also be induced by adsorption to the red cell surface of bacterial polysaccharides. Red cells exposed to streptococcal culture supernatants adsorbed a variety of streptococcal antigens, and antibodies to these were found to occur in normal human sera.

(e) Finally, alteration of the serum without transformation of the red cells is known to produce unusual haemagglutination reactions. Such a phenomenon, "bacteriogenic haemagglutination", was noted by Davidsohn and Toharsky (1940,

1942) who showed that diffusible products of a corynebacterium and a pseudomonas organism separately produced changes in human plasma and serum which result in panagglutination of normal red cells independently of T-agglutinins (see Neter, 1956).

Quite apart from bacterial activity however physical treatment, e.g. brisk aeration of plasma, may lead to subsequently abnormal haemagglutination reactions with normal red cells (Foote, personal communication). In addition, the presence of cold agglutinins and incomplete Rh antibodies in serum may complicate investigations of the panagglutination phenomenon (see Kabat, 1956).

A recent report by Caselitz and Stein (1953) on the Thomsen agglutination phenomenon is of interest but illustrates the confusion in this field. These authors found evidence of "T ferment" (transforming principle) in culture filtrates of Cl. welchii types A and F, Cl. septicum and Cl. butyricum. Panagglutinability was best induced by "sensitisation" of the red cells with culture filtrate at 2°C. for 2 hours. The red cells were then washed free of the filtrate, mixed with serum and left at 2°C. for 20 hr for panagglutination to take place.

Thereafter, the tests were held for 1 hr at room temperature to exclude false positives due to cold agglutination before being read. Results varied from one sample of human serum to another and from day to day with the same sample. Pan-agglutination was diminished at room temperature and results were complicated by haemolysis at 37°C. The "ferments" of Cl. welchii and Cl. septicum showed marked differences in heat stability and rapidity of action and were thought to be different substances. The factors were readily obtained in liver broth cultures, slowly produced in ascorbic acid broth and not produced in nutrient broth cultures. With the homologous serum as agglutinin, red cells of ox, horse, pig, rabbit but not rat were sensitised, i.e. rendered panagglutinable, by the clostridial filtrates. Attempts to demonstrate neutralisation of the T ferments with anti-gas-gangrene serum were inconclusive. RDE activity was noted in the Cl. butyricum cultures.

The phenomenon originally described by Thomsen was ascribed to enzymatic decomposition of the red cell surface. The phenomena observed by Caselitz and Stein are unlikely to be due to actual enzyme activity at 2°C.; the conditions described favour adsorption mechanisms which may have been involved.

It is now apparent that an investigation of any direct bacterial haemagglutinin must take into account many possible sources of error if misleading interpretations are to be avoided. Red cells may be agglutinated by many substances including salts of heavy metals, inorganic acids and bases, fatty acids, basic proteins and plant and tissue extracts. The haemagglutinating activity of lipid fractions of animal tissue extracts is attributed to the presence of lecithin-cholesteral complexes. Lecithin-ergosterol complexes in yeast extracts have similar haemagglutinating activity (Stone, 1946). Difficulties in interpretation are greatly increased when serum is introduced into haemagglutination tests, as indirect mechanisms of haemagglutination, including panagglutination, may occur. In addition, the presence in serum of blood group substances and other mucoproteins may invalidate experiments designed to demonstrate specific serological neutralisation of enzymes destroying blood group substances or virus receptors on red cells by antisera prepared against bacterial haemagglutinin. Bacterial cultures contain many biologically active substances which may compete for related substrates. The hitherto accepted methods of demonstrating RDE activity, for

example, using myxovirus to indicate loss of agglutinability of the treated red cells are open to the charge that blockage of virus receptors at the red cell surface may simulate true receptor destruction. The results of experiments designed with these pitfalls in mind may help to characterise the haemagglutinin of *Cl. welchii* more precisely and indicate its relationship to other products of the organism.

SECTION II : Materials & Methods

MATERIALS AND METHODS

Strains. The Cl. welchii strains included a laboratory stock strain (W) which had been subcultured in cooked-meat broth over many years. Other stock strains representing types A - F obtained from the National Collection of Type Cultures (N.C.T.C.) and numbered 6719, 3110, 3180, 8346, 8084 and 8081 respectively, are referred to here by their appropriate type letter, "strain A" referring to N.C.T.C. strain of Cl. welchii, type A, no. 6719. Stock strains obtained through the courtesy of Dr A.T. Willis of the Bacteriology Department, Leeds School of Medicine, are designated by a number and type letter preceded by the letter L; thus "L2A" denotes Leeds strain 2, type A.

Four reference strains of Cl. welchii type A were obtained from Dr Betty Hobbs, Food Hygiene Laboratory, Colindale: (i) F 2985/50, a non-haemolytic, heat-resistant strain of Hobbs' serological type 1, isolated in 1950 from cooked salt beef; (ii) F 1546/52, a non-haemolytic, heat-resistant strain of Hobbs' serological type 10, isolated in 1952 from roast meat; (iii) F 6599/59, a classical β-haemolytic strain, untypable by Hobbs' sera 1 - 13, isolated post mortem from a necrotic jejunum in 1959; and (iv)

F 4637/60, a β-haemolytic strain, untypable by Hobbs' sera 1 - 13, isolated from faeces. Untyped strains of Cl. welchii isolated in our department are designated by numbers only: Strains 032, 443, 485 and 754 are β-haemolytic Cl. welchii isolated from specimens of pus; strains 300, 401, 402, 500 and 906 are β-haemolytic strains isolated from faeces; strains 029 and 153 are typical food-poisoning strains isolated from faeces and food respectively during two separate outbreaks of Cl. welchii food-poisoning in Edinburgh. All of these strains were subcultured many times in cooked-meat broth. A numerical suffix, e.g. A_1, A_2, denotes a sub-strain developed during subculture. The suffix 'GP', as in A GP, indicates a guinea-pig-passaged strain. Cultures of strains were lyophilised when it was desired to retain their original characteristics but in many cases serial subcultures in cooked-meat broth, with frequent checks of purity on anaerobic blood agar plates, were made to encourage haemagglutinin production.

Culture media. These included nutrient agar, nutrient broth, peptone water, horse blood agar and horseflesh-digest medium (Mackie and McCartney, 1953). The peptone was Oxoid Bacteriological Peptone (L37). Cooked-meat

broth was prepared as recommended by Mackie and McCartney, except that nutrient broth was added to the cooked meat instead of peptone-infusion broth; liquid paraffin was not added.

Red-cell suspensions. Citrated human group-A blood was obtained each week from the Blood Transfusion Department, Edinburgh Royal Infirmary, and stored at 4°C. It was washed three times by centrifugation with physiological saline and the washed cells made up as a 1 per cent. suspension in saline. Blood showing haemolysis was discarded and blood was not normally used after more than 1 week in the laboratory refrigerator. Experiments confirmed that different batches of blood kept in the refrigerator for varying times up to 1 month did not lose their sensitivity to the *Cl. welchii* haemagglutinin. Red-cell suspensions prepared according to the method described by Dafaalla and Soltys (1951) could be kept for much longer than 1 week, but these were not used for routine tests. Blood of human groups B and O, and blood of various animals, including guinea-pig, sheep, fowl, ox, horse, rabbit and mouse, was also used. With the exception of rabbit blood, which occasionally tended to auto-agglutinate, these bloods formed stable suspensions in physiological saline.

Blood grouping antisera were obtained from the Edinburgh Blood Transfusion Department.

Virus suspensions. Newcastle disease virus (NDV), strain Herts., and influenza virus (PR8) were supplied in allantoic fluid by Dr. R.H.A. Swain of this department.

Buffers. Acetic acid and sodium acetate (Walpole, 1914), boric acid and borax (Holmes, 1943), citric acid and sodium citrate (Lillie, 1948), citric acid and sodium phosphate (McIlvaine, 1921), phosphate (Sorensen, 1909), and Tris-maleate (Gomori, 1948) were used. Alcoholic stock solutions of citrate buffers (Lillie, 1948) were not used.

pH determinations. Values were checked on a Pye 'Dynacap' pH meter.

Ultrasonic disintegrates. Bacterial suspensions were held in ice-cooled narrow tubes and disintegration by the ¼ in. (6 mm.) probe of an M.S.E.-Mullard ultrasonic disintegrator was allowed to proceed for 8 - 10 min. Adjustments of depth of probe and tuning were necessary during this period.

Source of Cl. welchii haemagglutinin. Horse blood agar cultures (37°C. for 48 hr) were suspended each in 10 ml. physiological saline. The resultant suspension is referred to as "whole

culture". Centrifugation at 3000 r.p.m. for 15 min. yielded a clear supernatant and a bacterial deposit. The latter, resuspended in saline to the original volume, is referred to as "resuspended bacterial deposit".

Haemagglutination tests. Doubling dilutions of culture suspension or supernatant in physiological saline were mixed with an equal amount of a 1 per cent. saline suspension of thrice-washed red cells. The tests were left on the bench and were read when the controls had settled (Salk, 1944). Test-tubes, 5 x 5/8 in. (7.5 x 1.3 cm.) were used and 0.5 ml. amounts were titrated.

Haemagglutinating unit. Haemagglutinins were titrated and the dilution corresponding to the last tube showing complete haemagglutination (+ + +) was considered to contain 1 haemagglutinating unit for the amount of red cells employed in the test.

Haemagglutination-inhibition tests. A standard haemagglutinating dose of haemagglutinin (*Cl. welchii*, PR8 virus or blood group-A antiserum, 2 - 4 haemagglutinating units) in 0.5 ml. of saline was added to each of a series of doubling dilutions of inhibitor in 0.5 ml. amounts of saline. The mixtures were allowed to stand for 15 min. at 20°C.; 0.5 ml. of red-cell suspension

was then added to each tube, thoroughly mixed and left to settle. In assessing the inhibitor content of culture fluids it was important to heat these fluids prior to the test in order to inactivate any haemagglutinin produced by the culture.

Demonstration of enzyme destroying blood group-A substance. This was demonstrated by testing the inhibitory activity for the blood group-A isoagglutinin of doubling dilutions of heat-inactivated medium containing 1 per cent. peptone (Oxoid, L37) before and after growth of *Cl. welchii* in the medium, using the technique described for haemagglutination-inhibition tests above. When culture fractions were to be tested for the presence of the enzyme destroying blood group-A substance, 0.5 ml. of each fraction was incubated at 37°C. for 2 hr with 0.5 ml. of a 1 per cent. solution of Oxoid peptone in 0.01 per cent. thioglycollic acid in 0.05 *M* Tris-maleate buffer at pH 5.9. Control tubes containing saline in place of the peptone substrate, and others containing saline in place of the culture fraction, were included.

Haemolysin tests. Serial two-fold dilutions of the material to be tested were made in 0.5 ml. amounts of physiological saline containing calcium

chloride 0.01 per cent. Volumes of 0.5 ml. of 1 per cent. washed human red cell suspensions were then added. The mixtures were shaken, incubated at 37°C. in a water bath for 1 hr and chilled overnight at 4°C. before final observations of the degree of haemolysis (complete, almost complete, definite, doubtful or negative) were made for each tube in comparison with a negative control.

<u>Tests of lecithinase activity</u>. Serial doubling dilutions of 0.5 ml. of the test sample were made in 0.5 ml. aliquots of saline containing calcium chloride 0.01 per cent. Volumes of 0.5 ml. of egg-yolk suspension were then added. The mixtures were shaken, incubated for 1 hr at 37°C. in a water bath and then chilled overnight at 4°C. Turbidity produced by lecithinase is readily apparent in these tests and, for comparative purposes, an arbitrary visual estimation of turbidity was recorded as ++++, +++, ++, +, ± and - for each tube in comparison with a negative control. Comparative lecithinase assays based on these tests closely parallelled the results obtained in the haemolysin tests. In some later tests of lecithinase activity, egg-yolk broth (Oxoid PM 6) was used instead of egg-yolk suspension as substrate; the egg-yolk broth was a good

indicator of lecithinase activity. Parallel tests of lecithinase activity were performed on some fractions by dropping aliquots (0.02 ml.) on to the surface of an agar medium containing egg-yolk and measuring the zones of opacity produced after incubation of these plates, as suggested by Sheldon, Moskowitz and Deverell (1959). The method gave results comparable with but not quite as sensitive as the tube tests.

Demonstration of red cell myxovirus receptor inactivation. A modification of the test used by Wickham (1956b) based on the 'quantitative titration' described by McCrea (1947) was used. Two strains of virus, Newcastle disease virus (NDV) and influenza virus (PR8) were employed. Doubling dilutions of Cl. welchii culture material were prepared in 0.5 ml. physiological saline, 0.5 ml. of 1 per cent. washed red cell suspension added to each tube and the series incubated under conditions of time and temperature described in the text. Thereafter, the supernates were removed from the sedimented red cells which were mixed with 0.5 ml. of saline containing 5 - 10 haemagglutinating doses of the appropriate virus preparation. The tests were allowed to settle at room temperature.

Panagglutination tests. The techniques are

described in detail in Section III, 14. (ii).

Capsule studies. Bacterial capsules were demonstrated by microscopic examination of culture suspensions in very thin wet films of India ink (Duguid, 1951).

Electron-microscopic observations were made on bacilli harvested from blood agar cultures as for the haemagglutination tests. The bacilli were washed twice by centrifugation and resuspension in fresh saline and then fixed by addition of formaldehyde to about 0.25 per cent. After overnight suspension in the formol-saline at 4°C., the bacilli were washed twice by centrifugation and resuspension in distilled water. A drop of the final suspension was placed on a carbon-stabilised collodion membrane and allowed to dry in a desiccator overnight. The specimens were shadow-cast with gold-palladium alloy (60:40) at an angle of 15° and were then examined in the electron microscope (Associated Electrical Industries, EM 6) at 50 kv.

Concentration of protein solutions. The method of Palmstierna (1960) was used employing a form of carboxymethyl cellulose ('Courulose'; British Celanese Ltd., Coventry). Protein solutions were held in cellophan sacs prepared from dialysis tubing ('Visking'; Viskase, Ltd.,

87a West Bow, Edinburgh) and compressed in beds of Courulose at 4°C. until the desired reductions in volume were obtained.

Determination of lethal activity. Screening tests for lethal activity were performed by intravenous injection of 0.1 - 0.4 ml. volumes of appropriate dilutions of dialysed toxin in physiological saline into batches of four white mice of approx. 20 g. weight. Results were recorded at 48 hr.

Ammonium sulphate precipitation. The methods recommended by Dixon and Webb (1958) were followed.

Column chromatography. Water-cooled jacketed glass columns of 1 cm. internal diameter were packed with de-aerated carboxymethyl (CM-) cellulose or diethylaminoethyl (DEAE-) cellulose suspended in the appropriate buffer solution. The column was eluted with buffer solution carrying an increasing molarity gradient of sodium chloride. The eluting solution was delivered to the column from a reservoir flask by a micropump ('Miniflow'; LKB, Stockholm 12, Sweden) through a 200 ml. mixing vessel the contents of which were continuously agitated by an electrically-operated magnetic stirrer. Fractions were collected in tubes mounted on an automatic

fraction collector (Locarte Co., 24, Emperor's Gate, London, S.W. 7). The molarity of the effluent solution with respect to sodium chloride at any stage in the elution procedure could be calculated using the formula:

$$\mathrm{Log}_{10} (C_1 - C_v) = \frac{-v}{2.3\ V} + \log (C_1 - C_o)$$

or,

$$C_v = C_1 - (C_1 - C_o) e^{-\frac{v}{V}}$$

where V = volume of mixing vessel

C_1 = concentration of sodium chloride in reservoir

C_o = initial concentration of sodium chloride in mixing vessel

v = volume of effluent

C_v = concentration of sodium chloride in effluent after volume v.

(see Alm, Williams and Tiselius, 1952).
Routine molarity values were more conveniently determined by reference to a standard graph based on the above formula.

<u>Absorption spectra</u>. Unicam SP 500 and SP 600 spectrophotometers with 1 cm. light paths were used.

<u>Vibrio cholerae filtrate</u>. This was 'Duphar' crude filtrate (N.V. Philips - Roxane, Amsterdam, Holland).

<u>Pancreatin</u>. The crude product was supplied by British Drug Houses Ltd., Poole, Dorset.

<u>Trypsin</u>. Two preparations of crystallised trypsin from bovine pancreas were used: Difco "Trypsin 1:250" was held in a stock solution containing 1 per cent. in 0.01 N hydrochloric acid. This was diluted 1 in 10 with 0.05 M Tris-maleate buffer at pH 8.2 immediately before use. Crystallised bovine pancreatic trypsin (Light and Co., Colnbrook, England), with a trypsin activity of 2000 units per mg., was also used in a similar manner.

<u>Lysozyme</u> was prepared from egg-white in the Department according to the method of Carter (1949). The crude crystalline product was used at a concentration of 0.1 per cent. in 0.05 M Tris-maleate buffer at pH 6.0.

<u>Lipase</u> (Wheat germ: Worthington) was obtained from Light and Co., Colnbrook, England. It was used at a concentration of 0.1 per cent. in 0.05 M Tris-maleate buffer at pH 7.5.

<u>Egg-yolk suspension</u>. Hen egg yolks, separated from the whites, were pooled and mixed with physiological saline to obtain a 20 per cent. suspension by volume. The suspension was filtered two times (with difficulty) through Whatman no. 1 filter paper and stored at 4°C.

<u>Egg-white substrate solution</u>. This was prepared by homogenising 100 ml. of hen egg-white

with 400 ml. physiological saline buffered with 0.005 M phosphate buffer at pH 7.2. Mixing was achieved without undue frothing by stirring the mixture with a magnetic stirrer for 2 hr in a beaker and occluding the access of air to the surface by placing the base of a second beaker within the first beaker and in contact with the surface of the mixture. The solution was then centrifuged free of particulate debris and the clear supernate stored at -40°C. The final product, analysed on my behalf by Dr I.W. Sutherland and Mr J. Caird, had a nitrogen content of 3.2 mg. per ml. and a hexosamine content of 67.2 µg. per ml.; dry wt. = 32 mg./ml.

Hydrolysed egg white. Equal volumes of egg-white substrate solution and 0.1 N sulphuric acid were mixed and incubated at 80°C. for 2 hr to liberate free sialic acid. This crude hydrolysed material was estimated to contain 75 µg. of free sialic acid per ml. as measured by the Warren assay and standardised against pure N-acetyl neuraminic acid. It was stored at -40°C. and was used as a positive standard in the neuraminidase assay procedure.

Extraction of bovine submaxillary mucoprotein. This was done as recommended by Curtain and Pye (1955). Bovine submaxillary glands were

obtained immediately _post mortem_ at the abattoir and frozen solid before being taken to the laboratory where they were dissected free of fat and connective tissue, mixed with crushed ice and minced in a mincing machine. The material was then homogenised in a Waring blender with about twice its weight of distilled water, centrifuged at 5000 g. for 20 min. and the supernate taken. This was adjusted to pH 4 with 5 _N_ acetic acid and the resulting precipitate removed by centrifugation as before. The supernate was next neutralised with 5 _N_ sodium hydroxide and dialysed against two changes of excess distilled water for 48 hr. The dialysed extract was then fractionated with methanol.

Methanol fractionation of bovine submaxillary extract. The dialysed extract was made 0.1 _M_ with respect to barium by adding solid barium acetate. Methanol, pre-cooled to -40°C., was then stirred into the solution to give an alcohol concentration of 64 per cent. (v/v). Centrifugation in the cold at 2500 g. for 15 min. yielded a precipitate which was subsequently dissolved in a 0.1 _M_ solution of disodium ethylenediaminetetra-acetic acid (EDTA) and dialysed for 60 hr against three changes of excess distilled water. The solution was then refractionated under identical

conditions except that a methanol concentration of 56 per cent. (v/v) was used on this occasion.

Blood group-A substance from red cells was obtained through the courtesy of Dr J. Koscielak at Professor W.T.J. Morgan's department, The Lister Institute, Chelsea Bridge, London. The blood group-A activity of this material was stated to amount to 10 - 20 per cent. of that of the standard A substance obtained from ovarian cyst material when compared in haemagglutination-inhibition tests, and 15 per cent. as measured by quantitative precipitation tests using anti-A rabbit serum. The sample thus contained about 20 per cent. of pure blood group-A substance. It was soluble in distilled water to a concentration of 0.3 per cent.

Sialic acid. A 30 mg. sample of N-acetyl neuraminic acid was generously supplied by Dr Lars Svennerholm, University of Gothenberg, Sweden. The material was more than 99 per cent. pure and was used as a standard in the development of the neuraminidase assay.

Neuraminidase assay. The procedure is described in detail in Section III, 11. (iii).

Orcinol reaction. The procedure of Long and Staples (1959), based on the method of Bohm, Dauber and Baumeister (1954), was followed. A

1 ml. volume of the test sample was mixed with 1 ml. of Bial's orcinol reagent (0.02 per cent. of ferric chloride and 0.2 per cent. of orcinol in 8 N hydrochloric acid). The mixture was heated on a boiling-water bath for 15 min., cooled and extracted with 4 ml. of *iso* amyl alcohol. After centrifuging, 3.5 ml. of the upper violet organic phase was transferred to a 1 cm. glass cell. Ethanol (0.05 ml.) was mixed with the contents to avoid turbidity caused by absorption of moisture. Extinctions were measured at wavelengths between 400 - 700 mμ. A standard positive and a reagent blank were simultaneously treated.

Thiobarbituric acid assay for sialic acid. The procedure, developed by Warren (1959), used the following solutions: (i) sodium *meta*periodate, 0.2 M in 9 M phosphoric acid; (ii) sodium arsenite, 10 per cent. in a solution of 0.5 M sodium sulphate in 0.1 N sulphuric acid; (iii) 2-thiobarbituric acid, 0.6 per cent. in 0.5 M sodium sulphate.

A 0.2 ml. amount of the test sample was mixed with 0.1 ml. of the periodate solution and held at room temperature for 20 min. Arsenite solution, 1 ml., was then added with shaking until a yellow-brown colour disappeared. The

thiobarbituric acid solution (3 ml.) was finally added and, after shaking, the tube was capped with a glass bead and heated in a boiling-water bath for 15 min. The heated mixture was subsequently cooled in cold water for 5 min. and then extracted with 4.3 ml. of cyclohexanone. Optical densities of the organic phase were routinely determined spectrophotometrically at a wavelength of 549 mμ using 1 cm. glass cells rinsed out with absolute alcohol. The procedure was also performed on 0.2 ml. of water for the blank vessel and on 0.2 ml. of hydrolysed egg-white solution of known free sialic acid content.

Precipitation of protein-bound sialic acid. When it was desired to use the orcinol reaction to measure liberated sialic acid in the presence of residual protein-bound sialic acid, the latter was precipitated by adding 0.5 ml. of a 4 per cent. solution of phosphotungstic acid in 1 N hydrochloric acid and the precipitate was removed by centrifugation. Addition of the chilled phosphotungstic acid solution to neuraminidase reaction mixtures and removal of protein by centrifugation prior to the Warren assay procedure was a convenient method of stopping the enzyme reaction in critical tests (Popenoe and Drew, 1957; Mayron, Robert, Winzler and Rafelson, 1961).

Commercial antisera. *Cl. welchii* type A and *Cl. oedematiens* type A diagnostic antitoxic sera containing cresol, 0.35 per cent., were supplied commercially by The Wellcome Research Laboratories, Beckenham, England.

Chemical reagents. All salts used were of analytic grade.

Glassware. Particular attention was paid to the cleanliness of all glassware used. Tubes were routinely cleaned by brushing out with cold water and then boiling for 30 min. in soap solution ('Lux' soap flakes, held as a stock 15 per cent. (w/v) aqueous solution which was diluted 1 in 30 for use). The tubes were thereafter brushed out in tap-water and boiled in clean water for 30 min. They were next rinsed three times in hot tap-water and then held in running tap-water for 45 min. The tubes were finally rinsed in 6 changes of cold tap-water and two changes of distilled water before being drained and dried in the hot-air oven.

SECTION III : Experimental observations

Production and demonstration of haemagglutinin

Demonstration of haemagglutinin. Experiments with eight strains of *Cl. welchii* tested in parallel using a microscope slide test (Gürtürk, 1952) , a porcelain tile test (Duguid *et al.*, 1955) and a tube test (Salk, 1944) showed that tube tests gave the most consistent results. Agglutination of red cells by the *Cl. welchii* haemagglutinin is much finer than the relatively coarse clumping described by Duguid *et al.* as typical of haemagglutination by fimbriate bacteria. Tile tests were positive when a strongly haemagglutinating bacterial deposit was used, but weaker concentrations of haemagglutinin were only detected by tube tests. Microscope slide tests using centrifuged "culture concentrates" as described by Gürtürk were not always as sensitive as tube tests, end-points were less clearly defined in haemagglutinin titrations and results were not consistent. Strain L2A, for example, grown in Gürtürk's medium and tested as centrifuged culture concentrate against a 1 per cent. suspension of fresh fowl red cells, gave weakly positive microscope slide tests in dilutions up to 1 in 24 whereas tube tests were clearly positive with dilutions of the same culture material up to

1 in 64. Table 2 summarises results representative of many experiments comparing the three types of test in parallel on the haemagglutinin content of 16 hr blood agar cultures harvested in 10 ml. saline and concentrated to 1 ml. by centrifugation.

Table 2

Comparison of a tile test, slide test and tube test for the demonstration of *Cl. welchii* haemagglutinin in 16 hr horse blood agar culture concentrate active against human red cells.

Strain of *Cl. welchii*	Tile test	Slide test	Tube test
A_1	-	-	-
B	+	++	+++
C	-	+	+++
D	-	-	-
E	+	++	+++
F	-	-	-
W	+	++	+++
443	-	-	-

- = no agglutination

+, ++, +++ = increasing degrees of agglutination.

The tile test and the slide test were not used routinely as they seemed insensitive and unreliable respectively. The tube test gave reproducible quantitative results. In tube test

titrations of serial doubling dilutions of *Cl. welchii* haemagglutinin, red cells settle in patterns very similar to those observed by Salk in virus haemagglutination tests. End-points are quite definite. Table 3 shows results obtained with five representative strains of *Cl. welchii*.

Table 3

Results of three types of haemagglutination test on 20 hr horse blood agar cultures of five strains of *Cl. welchii*, each harvested in 10 ml. physiological saline and tested against human red cells.

Strain of Cl.welchii	Haemagglutination Test						
	Tile Test	Slide Test	Tube test Culture diluted 1 in 4	8	16	32	64
A_2	-	++	+++	+++	+++	+	-
B	±	+++	+++	-	-	-	-
E	-	±	-	-	-	-	-
W	+	+++	+++	+++	+++	++	-
032	-	-	-	-	-	-	-

- = no agglutination; ± = doubtful positive;
+, ++, +++ = increasing degrees of agglutination.

Production of haemagglutinin. Different strains of Cl. welchii varied in the amount of haemagglutinin produced and in the time at which production was maximal. Table 4 shows the haemagglutinin production in blood agar cultures of eight strains grown for 1, 2, 4, and 8 days. Standard blood agar plates were inoculated as uniformly as possible with standard loopfuls of 48 hr cooked-meat broth cultures of the strains to be tested. Uninoculated plates of the same batch of blood agar were also incubated. All cultures were removed from the incubator, harvested in 10 ml. saline and tested as culture concentrate on the same day against human group-O red cells of the same batch. Washings of the incubated uninoculated plates were tested for any non-specific haemagglutinating activity and found to be negative.

The laboratory stock strain W produced large amounts of haemagglutinin. Titres were actually in excess of 1 in 128 for 24 and 48 hr blood agar culture concentrates and fell to 1 in 64 when 4 and 8 day cultures were tested. The N.C.T.C. strains of types A - E gave moderate to large amounts of haemagglutinin after 48 hr culture on blood agar at 37°C., but the type-F strain produced only a trace amount after 4 days. A recently isolated strain (443) similarly

produced negligible amounts of haemagglutinin.

Table 4

Haemagglutinin content of culture concentrates obtained from blood agar cultures of eight strains of Cl. welchii incubated for 1, 2, 4 and 8 days.

Strain of Cl. welchii	Age of culture (days)											
	1			2			4			8		
	diluted			diluted			diluted			diluted		
	1/4	1/8	1/16	1/4	1/8	1/16	1/4	1/8	1/16	1/4	1/8	1/16
A	±	-	-	++	-	-	+++	++	+	+++	+++	±
B	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+
C	++	±	-	+++	+++	±	±	-	-	±	±	-
D	-	-	-	+++	±	-	+++	+++	++	+++	+++	+
E	+++	+++	-	+++	+++	+++	+++	+++	+++	+++	+++	+++
F	-	-	-	-	-	-	+++	±	±	+++	+	-
W	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
443	-	-	-	+	-	-	++	±	±	±	-	-
Uninoculated control plates.	-	-	-	-	-	-	-	-	-	-	-	-

Guinea-pig passage of haemagglutinating strains caused a marked reduction in their ability to produce haemagglutinin in immediately subsequent cultures. For example, 2 ml. of a 48 hr blood agar culture suspension of strain L2A with a haemagglutinin titre of 1 in 128 was injected intramuscularly into the thigh of a guinea-pig. After 48 hr the strain was recovered from the oedematous limb and from the heart blood of the moribund animal. Haemagglutinin titres of 48 hr blood agar cultures of the passaged organism were 1 in 8 for the strain recovered from the oedema fluid and 1 in 2 for the strain recovered from the heart blood.

Freshly isolated strains of Cl. welchii never produced haemagglutinin. Over 100 new strains were obtained from human faeces and several more from wounds and other lesions; all were found inactive when tested in 48 hr blood agar cultures within a day or two of isolation. The ability to produce haemagglutinin developed in some of these strains after 4 - 5 months' cultivation involving several subcultures in cooked-meat broth. For example, strain 443 produced no haemagglutinin when originally isolated from an ear swab in February, 1958. First evidence of haemagglutinin production by this strain was noted in a 48 hr cooked-meat

broth subculture prepared in mid-June, 1958.

Although a limited investigation of stock strains of Cl. welchii obtained from two other laboratories confirmed the general impression that haemagglutinin production was typically associated with strains which had been subcultured in the laboratory for some time, examples of old non-haemagglutinating strains were noted. All of six stock strains, types A (3), C (1) and D (2), obtained from Dr A.T. Willis of Leeds University Medical School, produced haemagglutinin in 48 hr blood agar cultures but minimal amounts were produced by strains L1A and L9D. One of two stock β-haemolytic type-A strains obtained from Dr Betty C. Hobbs of the Food Hygiene Laboratory, Colindale, produced haemagglutinin. However, cultures of another typical type-A strain were virtually non-haemagglutinating and two typical food-poisoning strains, both of which had been subcultured many times in cooked-meat broth for several years, produced no haemagglutinin (Table 5).

Table 5

Haemagglutinin production in 48 hr horse blood agar cultures of various stock laboratory strains of Cl. welchii.

Strain	Haemagglutination with suspension diluted 1 in								
	2	4	8	16	32	64	128	256	512
L1A	+++	-	-	-	-	-	-	-	-
L2A	+++	+++	+++	+++	+++	+++	+++	+++	-
L3A	+++	+++	+++	+++	-	-	-	-	-
L7C	+++	+++	+++	+++	+++	+++	+++	+++	+++
L8D	+++	+++	+++	+++	+++	+++	+++	+++	-
L9D	++	+	±	-	-	-	-	-	-
F4637/60	+	-	-	-	-	-	-	-	-
F6599/59	+++	+++	+++	++	-	-	-	-	-
F1546/52	-	-	-	-	-	-	-	-	-
F2985/50	±	-	-	-	-	-	-	-	-

L1A, L2A, L3A, L7C, L8D, L9D - Leeds strains

F4637/60, F6599/59 - Colindale type-A strains

F1546/52, F2985/50 - Colindale food-poisoning strains.

- = no agglutination
± = doubtful positive
+, ++, +++ = increasing degrees of agglutination.

Effect of medium on haemagglutinin production. When Cl. welchii was grown for 48 hr in the media commonly used, blood agar cultures yielded the largest amount of haemagglutinin, both in centrifuged bacillary deposits and in bacteria-free supernates of culture suspensions. Cultures on normal, boiled or lysed blood agar media did not give significantly different yields. Nutrient agar and nutrient broth cultures produced less haemagglutinin and the latter were frequently haemolytic. Cooked-meat broth cultures at 48 hr gave varying amounts of haemagglutinin but usually less than blood agar cultures. For the maximum yield, most strains required either 48 - 72 hr growth on blood agar or 4 - 5 days' growth in cooked-meat broth. Cultures in Ellner's (1956) sporulation medium produced large amounts of haemagglutinin which, after centrifugation of the cultures, was almost entirely associated with the bacteria-free supernatant fluid. For example, bacteria-free washings from a 15 hr blood agar culture of strain W had a haemagglutinin titre of 1 in 32 compared with a titre of 1 in 128 for a centrifuged 15 hr Ellner culture supernate of the same strain. The resuspended washed bacterial deposit from the Ellner culture was non-haemagglutinating whereas washed bacterial suspensions obtained from blood

Table 6

Haemagglutinating/haemolytic activities of *Cl. welchii*, strain W, during culture for 6 days in tubes of nutrient broth containing varying proportions of cooked-meat broth supernate and tested as whole culture against human red cells.

Composition of medium		Age of culture (days)																				
		1							2							3						
		diluted 1 in							diluted 1 in							diluted 1 in						
Nutrient broth (ml.)	Cooked-meat broth supernate (ml.)	4	8	16	32	64	128	256	4	8	16	32	64	128	256	4	8	16	32	64	128	256
10	0	L	L	L	L	1 +++	±	-	+++	+++	+++	+++	+++	+	-	L	L	L	1 +++	1 ++	-	-
9.9	0.1	L	L	L	L	1 +++	±	-	+++	+++	+++	+++	+++	±	-	L	L	L	1 +++	+++	-	-
9.8	0.2	L	L	L	L	1 +++	±	-	+++	+++	+++	+++	+++	±	-	L	L	1 +++	+++	++	-	-
9.0	1.0	L	L	L	±	+++	±	-	-	-	+++	+++	+++	±	-	1 +++	1 +++	+++	+++	++	-	-
8.0	2.0	L	L	-	+++	+++	+++	-	-	-	++	+++	+++	++	-	1 +	+++	+++	+++	+++	+	-
5.0	5.0	-	-	-	++	++	+++	-	+	-	±	++	+++	+	-	++	+++	+++	+++	+++	-	-

L = complete lysis; 1 = incomplete lysis;

± = doubtful positive agglutination;

+, ++, +++ = increasing degrees of agglutination.

Table 6 continued:

Composition of medium		Age of culture (days)																				
		4							5							6						
Nutrient broth (ml.)	Cooked-meat broth supernate (ml.)	diluted 1 in							diluted 1 in							diluted 1 in						
		4	8	16	32	64	128	256	4	8	16	32	64	128	256	4	8	16	32	64	128	256
10	0	+++	+++	+	-	-	-	-	+++	+++	+	-	-	-	-	+++	+++	+	⊥	-	-	-
9.9	0.1	+++	+++	⊥	-	-	-	-	+++	+++	+	-	-	-	-	+++	+++	++	⊥	-	-	-
9.8	0.2	+++	+++	⊥	⊥	-	-	-	+++	+++	+	⊥	-	-	-	+++	+++	+	⊥	⊥	-	-
9.0	1.0	+++	+++	+++	⊥	-	-	-	+++	+++	+	⊥	-	-	-	+++	+++	+++	++	⊥	-	-
8.0	2.0	+++	+++	+++	⊥	-	-	-	+++	+++	+++	++	⊥	-	-	+++	+++	+++	+	-	-	-
5.0	5.0	+++	+++	+++	+	⊥	-	-	+++	+++	+++	+	-	-	-	+++	+++	+++	+++	+	⊥	-

⊥ = doubtful positive agglutination

+, ++, +++ = increasing degrees of agglutination

agar cultures (see Methods) retained considerable haemagglutinating activity (q.v.).

Haemagglutin production in nutrient broth cultures was not enhanced by the addition of red cells or red cell stromata to the medium. Addition to nutrient broth of the supernatant fluid from sterile cooked-meat broth was found to suppress the haemolytic activity of cultures grown for 1 - 3 days in this medium, but did not increase their haemagglutinating activity. Haemagglutination was also initially suppressed in low dilutions of those cultures containing significant amounts of cooked-meat broth supernatant, but this effect disappeared after the third day of culture (Table 6).

The addition of increasing amounts of cooked-meat to a series of tubes of sterile cooked-meat broth supernatant caused proportionate reduction in the haemagglutinin content of cultures subsequently grown in these media for 20 hr. This suggests that an inhibitor of the haemagglutinin is contributed to cooked-meat broth from the meat, and this inhibitor appeard to be inactivated on prolonged incubation with Cl. welchii.

Haemagglutinating activity was markedly reduced in cultures grown on blood agar containing either calcium chloride, 0.1 per cent., or glucose, 0.1 - 1.0 per cent. This occurred even when

calcium inhibitors were incorporated in the diluents of the haemagglutination tests to suppress the enhanced haemolytic activity of these cultures. A typical experiment showed a fourfold reduction in haemagglutinin production when glucose, 0.1 per cent., was added to blood agar. Virtually no haemagglutinating activity was observed in a similar culture of the same strain grown on blood agar containing calcium chloride, 0.1 per cent.

The haemolytic effect of some 24 hr Ellner broth cultures of haemagglutinating strains largely masked their haemagglutinin activity which was evident, however, at one or two doubling dilutions beyond the haemolysin titre. These cultures showed gradual loss of haemolysin when tested after further incubation. Haemagglutinin activity was maintained and, after 3 or 4 days, was apparent also at the low dilutions previously associated with haemolysis (Table 7).

These results suggest that lecithinase activity may interfere with haemagglutinin activity at the red cell surface. Some cultures with marked haemolysin activity showed a subsequent loss of lysin without at any time giving haemagglutination so that it is unlikely that the haemagglutinin is merely a degradation product of

the haemolysin. This was particularly evident with an Ellner culture of a guinea-pig passaged strain (A/GP). Haemolytic activity was demonstrable with this culture up to 138 hr, but haemagglutinating activity was never demonstrable when tested for at 22, 44, 90, 138 and 160 hours.

Table 7

Haemagglutinin and haemolysin production of thirteen strains of Cl. welchii grown in Ellner medium and tested as whole culture against human red cells. (All Ellner cultures were subcultured on anaerobic blood agar plates at 44 hr to check growth and purity. Strains 153 and F showed relatively poor growth.)

Strain	Age of culture																	
	22 hr						44 hr						90 hr					
	diluted 1 in						diluted 1 in						diluted 1 in					
	4	8	16	32	64	128	4	8	16	32	64	128	4	8	16	32	64	128
A	L	L	L	-	-	-	L	-	-	-	-	-	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-	L	L	1 +++	-	-	-
C	L	L	L	?+++	+++	-	L	L	L	+++	+++	+++	+++	+++	+++	+++	-	-
D	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	-
E	L	L	L	?+++	±	-	L	L	1	+	-	-	1 +++	1 +++	+++	+++	+	-
F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
W	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	-
443	L	L	L	+++	+++	+++	L	? 1 +++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
754	+++	+++	-	-	-	-	+++	±	-	-	-	-	±	-	-	-	-	-
032	L	L	L	L	+++	+++	L	L	1 +++	+++	+++	+++	L	1 +++	+++	+++	+++	+++
032GP	L-	L	+++	+++	+++	+++	L	+++	+++	+++	++	-	+++	+++	+++	+++	-	-
153	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A GP	L	L	L	L	L	L	L	L	L	L	L	1	L	L	L	L	L	1
Neg. control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

L = complete lysis
1 = partial lysis

+, ++, +++ = increasing degrees of haemagglutination
± = doubtful haemagglutination
- = no haemagglutination

Factors influencing the haemagglutination reaction

Effect of diluent in the haemagglutination test. Physiological saline was used routinely as the diluent for haemagglutinin titrations. Addition of 0.01 per cent. calcium chloride , 0.1 per cent. Versene or 0.08 per cent. sodium azide did not affect the haemagglutinin titre ; only slight inhibition occurred when 0.5 per cent. sodium oxalate, citrate or phosphate was added to suppress alpha-haemolysin .

Table 8 (overleaf) shows the results of an experiment in which the effects of some of these substances were studied when used in the diluent of parallel tests of a haemagglutinating strain (W) grown on blood agar and in glucose broth and tested against sheep red cells. The sheep red cells were added to each titration as a suspension in the diluent under test.

Table 8

The influence of various substances on the haemagglutinating and haemolytic activity of blood agar and glucose broth cultures of *Cl. welchii* tested in parallel against sheep red cells.

Test	1		2		3		4		5	
Diluent	Form aldehyde 0.5 per cent.		Sod. citrate 0.5 per cent.		Sod. phosphate 0.5 per cent.		CMB supernatant undiluted		Saline (0.85 per cent. sod. chloride)	
Medium	BA	GB	BA	GB	BA	GB	BA	GB	BA	GB
Cultures diluted 1 in: 2	+++	L	+++	1	+++	-	-	-	+++	L
4	+++	L	+++	-	+++	-	-	-	+++	L
8	+++	L	+++	-	+++	-	-	-	+++	L
16	+++	-	+++	-	++	-	-	-	+++	-
32	+	-	++	-	⊥	-	-	-	++	-
64	-	-	⊥	-	-	-	-	-	-	-
128	-	-	-	-	-	-	-	-	-	-
Negative red cell controls	-	-	-	-	-	-	-	-	-	-

L = complete lysis
1 = partial lysis

+, ++, +++ = increasing degrees of haemagglutination
⊥ = doubtful haemagglutination
- = no haemagglutination

Sodium citrate, 2 per cent. in saline, reduced the haemagglutinin titre. The pH of this solution was 8.4 and the inhibition was considered to be a pH effect. (q.v.)

Formaldehyde, 0.5 per cent., in the saline diluent reduced the haemagglutinin titre slightly but not the haemolysin titre. The use of the supernatant of sterile cooked-meat broth as diluent suppressed both haemagglutination and haemolysis. These results suggest that, while haemolysis can mask haemagglutinin activity, haemagglutinin is not produced in significant amount in glucose broth cultures and the haemagglutination phenomenon is not significantly sensitive to chelating agents.

Effect of temperature of haemagglutination test. In tube tests Cl. welchii haemagglutinin acted equally well in the cold (4°C.) and at room temperature (20°C.). Tile tests at 37°C. showed macroscopic agglutination appearing within 3 - 4 min. and becoming maximal in 10 - 15 min. At 20°C. the reaction was slower, and at 4°C. it required twice as long as at 37°C. Tube tests at 37°C. were frequently complicated by irregular elution of the haemagglutinin and haemolysis.

Effect of pH of the test. The haemagglutinating activities of whole culture, bacterial deposit and culture supernatant were separately

tested at pH values ranging from 2.2 to 9.4. Tests at values below 5.6 were unreliable owing to changes in the red cells, but distinct haemagglutination occurred in mixtures ranging from pH 5.6 to 9.4. Tube tests of whole culture of strain L2A diluted in Tris-maleate buffered saline showed a broad range of optimal activity from pH 6.0 to 7.5 (Table 9).

Table 9

The effect of pH on the activity of *Cl. welchii* haemagglutinin in whole culture against human group-A red cells.

pH of test	Haemagglutination with culture diluted 1 in				
	4	8	16	32	64
5.6	+++	+++	+++	±	-
6.0	+++	+++	+++	+++	-
6.8	+++	+++	+++	+++	-
7.5	+++	+++	+++	+++	±
8.2	+++	+++	+++	±	-
Saline control	+++	+++	+++	+++	-

- = no agglutination;

± = doubtful positive;

+, ++, +++ = increasing degrees of agglutination.

Tube tests of culture supernatant of strain A showed maximum titres at pH 5.6 and 6.8 (citrate-phosphate buffers), but these titres were not significantly superior to those obtained in unbuffered saline (Table 10).

Table 10

The effect of pH on the activity of *Cl. welchii* haemagglutinin in culture supernatant fluid against human group-A red cells.

pH of test	Haemagglutination with supernatant diluted 1 in				
	4	8	16	32	64
5.4	+++	-	-	-	-
5.6	+++	+++	++	+	-
5.8	+++	+++	-	-	-
6.4	+++	+++	++	-	-
6.8	+++	+++	+++	-	-
7.2	+++	++	-	-	-
7.7	+++	+++	-	-	-
saline control	+++	+++	+++	-	-

- = no agglutination;

+, ++, +++ = increasing degrees of agglutination

In tile tests of 3 day whole blood agar culture

suspension of strain L2A at 20°C., using Tris-maleate buffers, haemagglutination of human red cells occurred most rapidly at pH 6.8 (Table 11). These tests were done 'blind' and in duplicate to eliminate observer error, but results were unequivocal.

Table 11

Effect of pH on the rate of development of haemagglutination in duplicate tile tests of *Cl. welchii* haemagglutinin in whole culture against human group-A red cells at 20°C.

Time (min.)	pH of test										saline controls	
	5.6		6.0		6.8		7.5		8.2			
1	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	±	±	-	-	-	-	-	-
6	±	-	±	-	+	+	±	±	±	±	+	+
7½	±	±	+	±	++	+	+±	±	±	±	++	++
9	±	±	+±	+	++	++	+±	+	+	+	++	++
11	±	±	+±	+	+++	+++	+±	+	+±	+	+++	+++
15	+	+	++±	++	+++	+++	++	++	++	+	+++	+++

- = no agglutination
± = doubtful positive
+, +±, ++, ++±, +++ = increasing degrees of agglutination.

Range of cells agglutinated. The haem-agglutinin was strongly and equally active with human red cells of all ABO groups. The red cells of rabbit, guinea-pig, fowl, sheep, ox, mouse and horse were found decreasingly sensitive in this order, those of mouse and horse being relatively insensitive. Bovine (ox) red cells varied in sensitivity, some samples reacting almost as strongly as human red cells, but in general they were not particularly sensitive to the haemagglutinin. Typical results are illustrated by the experiment summarised in Table 12 in which relatively insensitive ox cells were encountered.

Table 12

A comparison of the sensitivity of red cells of various species (1 per cent. saline suspensions) to the haemagglutinin of Cl. welchii strain A (48-hr harvested blood agar culture supernatant).

Species of red cell	Culture supernatant diluted 1 in					
	4	8	16	32	64	128
Human group-A	+++	+++	+++	++	⊥	-
Human group-B	+++	+++	+++	++	-	-
Human group-O	+++	+++	+++	++	⊥	-
Rabbit	+++	+++	+++	++	⊥	-
Guinea-pig	+++	+++	++	⊥	-	-
Fowl	+++	+++	⊥	-	-	-
Sheep	+++	+++	+	-	-	-
Ox	+	-	-	-	-	-
Mouse	+++	-	-	-	-	-
Horse	++	⊥	-	-	-	-

- = no agglutination
⊥ = doubtful positive
+, ++, +++ = increasing degrees of agglutination.

Bovine red cells became much more sensitive if treated with formaldehyde by the method of Dafaalla and Soltys (1951). For example, harvested 48 hr blood agar culture of strain W produced haemagglutination in dilutions up to 1 in 8 when tested with formalinised ox cells whereas agglutination only occurred at a 1 in 2 dilution when untreated ox cells were used. The incorporation of rabbit serum in the tests as recommended by Dafaalla and Soltys did not enhance agglutination and, as the presence of serum added further complications in the interpretation of results (q.v.) it was not routinely used. Human red cells were not rendered more sensitive when treated with tannic acid as described by Boyden (1951), but gave stable suspensions of exceptionally high sensitivity when treated with V. cholerae filtrate (RDE) until no longer agglutinable by influenza PR8 virus (Table 13) . Guinea-pig leucocytes were agglutinated by the haemagglutinin.

Properties of the haemagglutinin

Stability. The haemagglutinating activity of whole culture or centrifuged culture supernatant diminished slowly and irregularly on standing for several days at 4°C., 20°C., or 37°C. Transient increases in activity of clear culture supernatants were occasionally detected during

Table 13

The effect of RDE-treatment of human group-A red cells on their sensitivity to *Cl. welchii* haemagglutinin derived from three different strains.

Haemagglutinin derived from *Cl. welchii* strain	Normal red cells exposed to culture supernate diluted 1 in						RDE-treated red cells exposed to culture supernate diluted 1 in							
	4	8	16	32	64	C	4	8	16	32	64	128	256	C
A	+++	+++	+++	++	-	-	+++	+++	+++	+++	+++	++	+	-
D	+++	+++	++	+	-	-	+++	+++	+++	+++	+++	++	⊥	-
B	⊥	-	-	-	-	-	++	+	-	-	-	-	-	-

- = no agglutination; ⊥ = doubtful positive;
+, ++, +++ = increasing degrees of agglutination.

storage at 20°C. and 4°C. The haemagglutinin in culture supernatant was not inactivated by incubation at 37°C. for 24 hr at pH values ranging from 5.6 to 8.2, but the activity of unbuffered bacterial suspensions and culture supernatants was destroyed after 30 - 45 min. at 55°C., after 5 - 10 min. at 60°C. or after 1 min. at 100°C. Buffered preparations of whole culture of strain L2A (Tris-maleate buffers) showed least rapid inactivation at an alkaline pH (Table 14).

Table 14

The effect of pH on heat-inactivation times of haemagglutinin in whole culture of *Cl. welchii* strain L2A.

Minutes of heating at 55°C.	Haemagglutination after heating					
	at pH					unbuffered saline controls
	5.6	6.0	6.8	7.5	8.2	
0	+++	+++	+++	+++	+++	+++
10	-	++	+++	+++	+++	+++
20	-	-	+	±	+++	+++
30	-	-	-	+	++	+++
40	-	-	-	-	±	+
50	-	-	-	-	-	-

- = no agglutination
± = doubtful positive
+, ++, +++ = increasing degrees of agglutination.

The haemagglutinating activity of culture supernatant was not reduced by oxygenation and was not enhanced when tests were performed under reducing conditions. These results indicate that the haemagglutinin is thermolabile but not oxygen-sensitive.

The haemagglutinating activity of bacterial deposit was destroyed by treatment for 18 hr at 20°C. with chloroform, 1 per cent. phenol, or 1 per cent. formaldehyde, but not with 95 per cent. ethanol. The effect of formaldehyde was investigated in detail. Incubation of whole culture at 37°C. with 2 per cent. formaldehyde for 4 hr or 0.12 per cent. formaldehyde for 24 hr inactivated the haemagglutinin. Addition of 0.2 - 0.5 per cent. formaldehyde to the saline diluent used in the tests reduced the titre slightly but 1 per cent. formaldehyde reduced it fourfold. An equal volume of 2 per cent. formaldehyde in saline was added to tubes containing red cells already agglutinated by Cl. welchii and the cells were dispersed by shaking; they re-settled in negative patterns. Thus the haemagglutinin can be inactivated by formaldehyde in sufficient concentration, and it appears to be susceptible to inactivation even after haemagglutination has occurred.

Haemagglutinin was not inhibited or destroyed when mixed and incubated with the whole culture of

a non-haemagglutinating Cl. welchii strain prior to the addition of red cells. It was unaffected by treatment with lysozyme or lipase, partly inactivated by pancreatin at pH 8.2 in 2 hr at 37° C., and almost completely destroyed by trypsin under these conditions. It therefore seems that although the haemagglutinin is susceptible to enzymic action, the inactivity of cultures of non-haemagglutinating strains is not due to the presence of haemagglutinin-inactivating factors in these cultures.

Diffusibility. Blood agar cultures of strain A were examined at different times and the amount of diffusible haemagglutinin compared with that which remained associated with the bacterial deposit. The cultures were harvested in 10 ml. of saline and centrifuged to obtain clear supernatant and bacterial deposit. The latter was resuspended in 1 ml. of saline, i.e. one tenth the volume of the supernatant fluid, and the haemagglutinin titres of both determined. The results (Table 15) indicate that significant haemagglutinin production occurs in cultures after 12 hr incubation at 37° C. Diffusible haemagglutinin production was most evident at 41 hr.

Table 15

Diffusible and non-diffusible haemagglutinin associated with centrifuged supernatant (S) and tenfold concentrated bacillary deposit (BD) obtained by harvesting blood agar cultures of *Cl. welchii* strain A at different times.

Age of culture (hr)	Fraction tested: Culture Supernate or Bacillary deposit	Haemagglutination observed at a dilution of 1 in					
		2	4	8	16	32	64
7	S	-	-	-	-	-	-
	BD	+	±	-	-	-	-
12	S	-	-	-	-	-	-
	BD	+++	++	±	-	-	-
17	S	±	-	-	-	-	-
	BD	+++	+++	+++	++	++	±
41	S	+++	+++	+++	++	-	-
	BD	+++	+++	+++	+++	++	-
65	S	+++	+++	±	-	-	-
	BD	+++	+++	+++	++	++	
89	S	+++	+++	+++	±	-	-
	BD	+++	+++	+++	+++	+	-

Key as in previous Table.

This pattern was less clearly demonstrated with strain C. A virtually non-haemagglutinating strain (032) showed minimal activity demonstrable with the concentrated bacillary deposit only. Parallel capsulation studies using India ink wet films (Cruickshank _et al_., 1960) showed no relationship between capsule formation and haemagglutinin production (Table 16).

Table 16

Capsule formation and haemagglutinin production by blood agar cultures of three strains of _Cl. welchii_.

Age of culture (hr)	Strain of _Cl. welchii_		
	A	C	032
17	++ CC	+ c	- C
41	+++ CCC	+ C	± CC
65	++ CCC	++ c	+ CC
89	++ CCC	+ c	+ CC

- = no haemagglutinin
± = doubtful positive
+, ++, +++ = increasing amounts of haemagglutinin

c = minimal capsule formation
C, CC, CCC = increasing degrees of capsule formation.

In general, bacteria-free supernatant of centrifuged 20 hr culture was almost as strongly

haemagglutinating as whole-culture suspension . The resuspended bacterial deposit retained considerable activity and this could be further reduced by successive washings involving repeated centrifugation and resuspension in fresh saline. From 7 to 10 washings with 10 ml. amounts of saline were usually necessary to remove all activity from the bacterial cells (original haemagglutinin titre of cell suspension about 1 in 32). Fig. 1 illustrates an experiment in which the haemagglutinin titres of whole culture, supernatant and resuspended bacterial deposit were initially equal. As haemagglutinin was washed from the bacterial cells it was demonstrated in decreasing amounts in the bacteria-free centrifuged supernatants of the successive washings.

Release of cell-bound haemagglutinin by ultrasonic disintegration . Ultrasonic treatment of culture supernatant did not alter its haemagglutinating effect, but ultrasonic disintegration of the unwashed bacterial deposit greatly increased its haemagglutinating

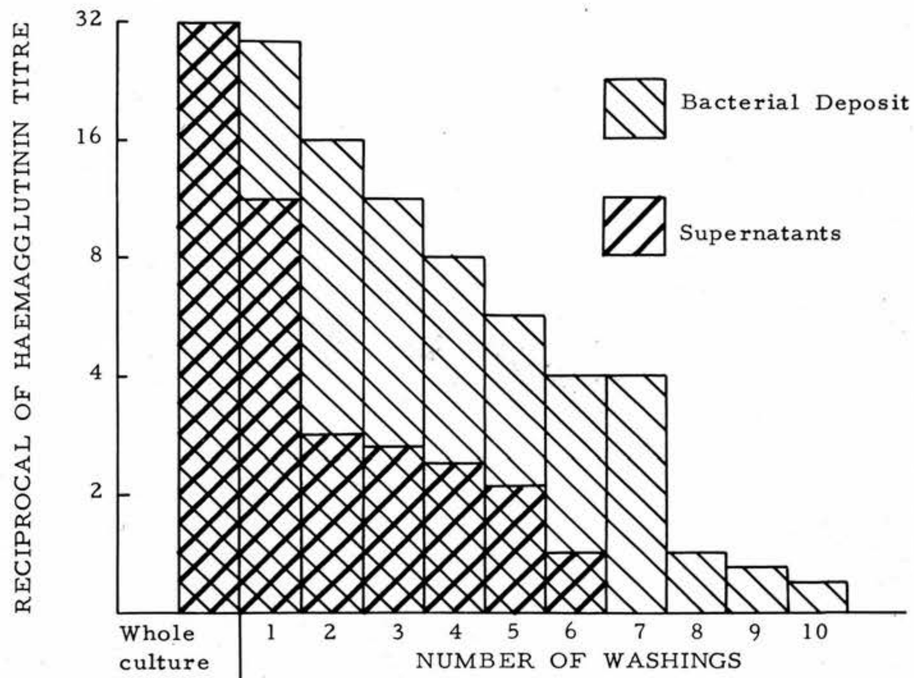


Fig. 1. Successive loss of Cl. welchii haemagglutinin from bacterial deposit following repeated washing by centrifugation and resuspension in fresh saline.

(Modified from Collee, 1961).

activity. This effect was not obtained by simple prolonged shaking of the bacterial deposit in saline even if this was performed in an atmosphere of hydrogen lest aeration produced inactivation of haemagglutinin. Ultrasonic disintegration of bacterial deposit which had been washed almost free of detectable haemagglutinin by 7 changes of saline, was found to release a large amount of haemagglutinin; this presumably had been contained intracellularly. Disintegration of the bacterial deposit of a non-haemagglutinating strain did not yield haemagglutinin. The following two Tables (17 and 18) summarise results of experiments upon which the above observations are based.

Ultrasonic disintegration of bacterial deposit provided a means of obtaining more concentrated haemagglutinin relatively free from diffusible haemolysins. Table 19 shows the results of an experiment in which an 18 hr "Oxoid" cooked-meat broth culture of strain A was tested against human red cells. When the whole culture or its centrifuged supernatant was used, haemolysis was troublesome. The haemagglutinating activity of the resuspended bacterial deposit was rather weak. Ultrasonic disintegration of the whole culture did not affect its haemolytic activity, but disintegrated bacterial deposit was non-haemolytic and powerfully haemagglutinating.

Table 17

Results of three separate experiments to show the effect of ultrasonic treatment in liberating haemagglutinin from bacterial deposit. Haemagglutinin was not liberated by shaking and could not be obtained by ultrasonic treatment of bacterial deposit of a haemagglutinin-negative strain.

Material tested	Diluted 1 in					
	4	8	16	32	64	128
Whole culture	+++	+++	+++	-	-	-
Culture supernatant	+++	+++	++	-	-	-
Culture supernatant after ultrasonic treatment.	+++	+++	+++	-	-	-
Resuspended bacterial deposit	+++	±	-	-	-	-
Resuspended bacterial deposit after ultrasonic treatment	+++	+++	+++	+++	+++	+++
Whole culture	+++	+++	+++	-	-	-
Whole culture after shaking (MICKLE 5 min.) in air.	+++	+++	+++	-	-	-
Whole culture after shaking in hydrogen	+++	+++	+++	-	-	-
Whole culture after ultrasonic treatment	+++	+++	+++	+++	+++	-
Whole culture of haemagglutinin-negative strain O32	-	-	-	-	-	-
Whole culture of strain O32 after ultrasonic treatment	-	-	-	-	-	-

Key: see Table 18.

Table 18

The release of cell-bound haemagglutinin by ultrasonic treatment of resuspended bacillary deposit (RBD) from which almost all detectable superficial haemagglutinin had been removed by washing.

Material tested	Diluted 1 in							
	2	4	8	16	32	64	128	256
Culture supernatant	+++	+++	+++	+++	+++	+	-	-
Resuspended bacillary deposit (RBD)	+++	+++	+++	+++	+++	±	-	-
RBD washed x 1	+++	+++	++	-	-	-	-	-
RBD washed x 4	+++	++	+	-	-	-	-	-
RBD washed x 7	++	-	-	-	-	-	-	-
RBD washed x 7 then disintegrated ultrasonically	+++	+++	+++	+++	+++	+++	-	-

- = no agglutination

± = doubtful positive

+, ++, +++ = increasing degrees of agglutination.

Table 19

The release of cell-bound haemagglutinin free from diffusible haemolysin by ultrasonic treatment of bacillary deposit.

Material tested	Diluted 1 in								
	2	4	8	16	32	64	128	256	512
Whole culture	L	L	L	L	L	-	-	-	-
Culture supernatant	L	L	L	L	l -	l -	-	-	-
Resuspended bacillary deposit	+++	+++	+++	-	-	-	-	-	-
After ultrasonic disintegration									
Whole culture	L	L	L	L	L	l +++	+++	++	-
Culture supernatant	L	L	L	L	L	l +	-	-	-
Resuspended bacillary deposit	+++	+++	+++	+++	+++	+++	++	±	-

L = complete lysis

l = partial lysis

+, ++, +++ = increasing degrees of haemagglutination.

± = doubtful haemagglutination

- = no haemagglutination

<u>Filtration studies</u>. Filtration of culture supernatant through sintered glass filters (grade 3, pores 20-30μ) did not significantly reduce the haemagglutinin content, but subsequent filtration through a grade 5 filter, pores up to 2μ, markedly reduced it; filtration was very slow. Table 20 summarises results of a typical experiment.

Table 20

The effect of filtration through different grades of sintered glass filters on the haemagglutinating activity of culture supernatant.

Material tested	Diluted 1 in				
	4	8	16	32	64
Culture supernatant	+++	+++	+++	±	-
After filtration through grade-3 sintered glass filter.	+++	+++	++	-	-
After filtration through grade-5 sintered glass filter.	+++	++	-	-	-

++, +++ = increasing degrees of haemagglutination

± = doubtful haemagglutination

- = no haemagglutination

Seitz asbestos filters absorbed the haemagglutinin completely and added a further

complication by shedding material which was shown to cause coarse clumping of red cells. Thus, Seitz-filtered saline diluted 1 in 2 with physiological saline agglutinated red cells moderately (++). The same Seitz-filtered saline was more actively haemagglutinating (+++ at a dilution of 1 in 2) after agitation. The supernatant fluid after centrifugation was non-haemagglutinating but the concentrated deposit obtained from about 8 ml. and resuspended in about 1 ml. of fluid was actively haemagglutinating (+++) in dilutions up to 1 in 8.

Filtration of actively haemagglutinating whole-culture suspensions through cellulose acetate membrane filters (Oxoid) very markedly reduced their haemagglutinin content. The haemagglutinin was not diffusible through cellophan when dialysed against excess physiological saline at room temperature or against distilled water at 4°C. overnight.

The results of preliminary ultracentrifuge studies indicate that the haemagglutinin may be associated with particles of the order of size of 10 - 20 mμ.

It is therefore clear that the Cl. welchii haemagglutinin remains active when in solution and free from the bacterial cell. This evidence supports Wickham's contention that the substance is at least partly diffusible. The presence of an inhibitor of the haemagglutinin in cooked-meat broth may explain the fact that Dafaalla and Soltys (1953) could detect haemagglutinin in concentrated bacterial deposits but not in the bacteria-free supernates of cultures of Cl. welchii grown in this medium . Dafaalla and Soltys therefore regarded the haemagglutinin as non-diffusible . The rapid absorption of haemagglutinin by Seitz asbestos accounts for the lack of haemagglutinating activity of the culture filtrates examined by Gürtürk (1952) who assumed that the haemagglutinin was non-diffusible. However, as 'diffusible' haemagglutinin occurs late in cultures, at a stage well beyond the exponential phase of growth, it is most probable that the haemagglutinin is not truly diffusible and is liberated as a result of autolysis of the bacterial cell . The release of additional haemagglutinin by disintegration of washed bacterial cells, and the occurrence of large yields of diffusible haemagglutinin in sporing cultures in Ellner medium , tend to confirm this view .

Electron microscopy. Careful examination of washed bacterial deposits from non-haemagglutinating and actively haemagglutinating cultures by the electron microscope showed the absence of fimbriae. Many fields were searched and typical bacilli are illustrated (Plate 2). The appearances are those of a capsulate organism, the shrunken capsule obscuring the marginal line of the cell-wall.

The occurrence of peripheral artefacts is noteworthy. Bacteria surrounded by these apparently mucinous strands were common in preparations of haemagglutinating and non-haemagglutinating strains. They seem to be projections of capsular material, the main proportion of which is contracted around the bacillus during dehydration. In their continuity with the capsule, their frequent terminal bifurcation, their tendency to confluence, irregular breadth and haphazard arrangement, these projections are readily distinguishable from true fimbriae (Plate 1).

Electron microscopy of the concentrated supernatant fluid obtained from 48 hr Ellner cultures of strain L2A (haemagglutinin titre 1 in 1024) and a non-haemagglutinating freshly isolated control strain was kindly performed by

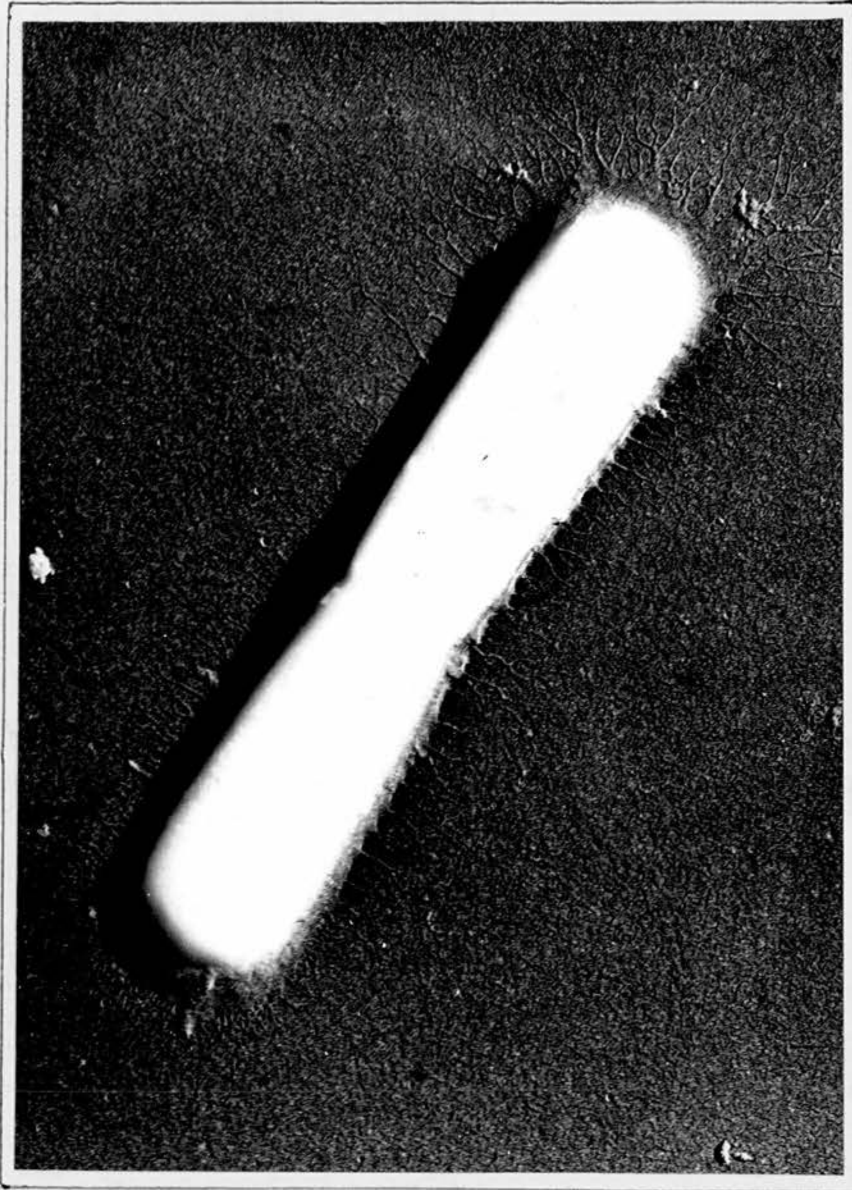


Plate 2. Clostridium welchii : a dividing cell from a 48-hr culture of a haemagglutinating strain. The absence of fimbriae and the presence of peripheral strands of capsular material are typical. Electron micrograph X 29,000.

Dr A.P. Waterson of Cambridge University. Dr Waterson reported that the material from the haemagglutinating culture contained numerous small particles about 500 Å (50 mμ) in diameter with no structure to be resolved but which might have a sub-unit arrangement. This small material was missing in the haemagglutinin-negative control specimen.

Adhesion experiments. The haemagglutinin associated with the bacilli in cultures of *Cl. welchii* does not appear to enable them to adhere to cell surfaces. Haemagglutinating and non-haemagglutinating bacilli were examined in wet films by phase-contrast microscopy for adhesion to various kinds of cells. It was occasionally possible to demonstrate adhesion of *Cl. welchii* to red cells but this was the exception rather than the rule.

Adhesion tests using cultures of varying ages and a wide range of cells including human red cells of all ABO groups, human buccal epithelium, red cells of various animals, guinea-pig epithelial cells from trachea and small and large gut and guinea-pig leucocytes, have shown no significantly consistent attachment of

haemagglutinating Cl. welchii to these cells. Adhesion was similarly negative in the case of Candida albicans cells and plant root hairs. Haemagglutinating bacilli injected intramuscularly into a guinea-pig showed no obvious tendency to adhere to the cells of the resulting exudate. It was found, however, that Cl. welchii organisms could agglutinate and adhere to particles of Seitz asbestos, Fuller's Earth and charcoal. This phenomenon was first observed with haemagglutinating strains but was shown also by non-haemagglutinating strains and is not caused by the haemagglutinin.

Adsorption and elution. Charcoal, Seitz asbestos, calcium phosphate gel and kieselguhr readily adsorbed the haemagglutinin which was also rapidly adsorbed, within minutes at 4°C. or 20°C., on to susceptible red cells. It was not so readily adsorbed by horse red cells, which are relatively insensitive to the haemagglutinin. Absorption of a haemagglutinating culture supernate with human red cells removed agglutinin simultaneously for human, ox, sheep and fowl red cells. When the centrifuged red cell deposits obtained from 10 ml. of 1 per cent. washed suspensions of red cells of man, ox, sheep or fowl were separately resuspended in 2.2 ml. aliquots of haemagglutinating culture supernate and

subsequently removed by centrifugation after 30 min. at bench temperature, haemagglutinating activity of the culture supernate was lost in each case for all of the range of red cells tested (Table 21).

Table 21

The haemagglutinating activity of aliquots of a culture supernate of *Cl. welchii*, strain L2A, before and after absorption of haemagglutinin by red cells of various species.

UNABSORBED	TESTED AGAINST RED CELLS OF	Culture supernatant diluted 1 in 4	8	16	32	64	128
	MAN	+++	+++	+++	+++	+++	-
	OX	+++	+++	+++	+++	++	-
	SHEEP	+++	+++	+++	-	-	-
	FOWL	+++	+++	+++	+++	-	-
ABSORBED WITH RED CELLS OF	**TESTED AGAINST RED CELLS OF**	**Absorbed Culture supernatant diluted 1 in 4**	**8**	**16**	**32**	**64**	**128**
MAN	MAN	-	-	-	-	-	-
	OX	-	-	-	-	-	-
	SHEEP	-	-	-	-	-	-
	FOWL	-	-	-	-	-	-
OX	MAN	-	-	-	-	-	-
	OX	-	-	-	-	-	-
	SHEEP	-	-	-	-	-	-
	FOWL	-	-	-	-	-	-
SHEEP	MAN	-	-	-	-	-	-
	OX	-	-	-	-	-	-
	SHEEP	-	-	-	-	-	-
	FOWL	-	-	-	-	-	-
FOWL	MAN	+++	+	-	-	-	-
	OX	±	±	-	-	-	-
	SHEEP	-	-	-	-	-	-
	FOWL	+++	++	-	-	-	-

Control tests with saline washings from the original washed red cells showed that no inhibitor was released from these red cells in this experiment. The red cells employed to absorb the haemagglutinin showed little subsequent release of haemagglutinin into saline or Tris-maleate buffered saline over a pH range of 5.6 to 7.5 during 30 min. at 37°C. although fowl red cells, which had not absorbed haemagglutinin as completely as the other cells tested, yielded some of their adsorbed haemagglutinin more readily.

When fowl red cells with adsorbed haemagglutinin were washed once in the cold to separate them from free haemagglutinin and then mixed with normal human red cells, some clumping of the human cells was seen with the phase-contrast microscope; in addition, human red cells became adherent to clumps of fowl red cells, forming mixed clumps with them. This suggests that bivalent adhesive haemagglutinin had remained bound to the surface of the fowl red cells and that the action of the Cl. welchii haemagglutinin is not merely to render cells auto-agglutinable by destruction of surface components.

Red cells agglutinated by Cl. welchii haemagglutinin and subsequently shaken and held at 37°C. tended to settle in a negative pattern, but when the cells were again dispersed and returned

to the bench at 20°C. they settled in their original agglutinated pattern, sometimes showing a slight rise in haemagglutinin titre. This agglutination and elution procedure could be repeated several times with the same test over the course of 2 days. The cells always tended to show negative patterns at 37°C., but re-agglutinated on removal to bench temperature, although there was then a gradual and successive reduction in titre (Table 22).

Table 22

The effect of alternate changes of incubation temperature on the haemagglutination pattern observed in a serial dilution test agitated prior to each change of temperature.

Day	Temperature during settling (°C.)	Culture diluted 1 in						Red cell controls in saline
		4	8	16	32	64	128	
1	20	+++	+++	+++	+++	-	-	-
	37	-	++	±	±	-	-	-
	20	+++	+++	+++	+++	+++	-	-
	37	-	-	-	-	-	-	-
	20	+++	+++	+++	+++	+++	-	-
	37	-	-	-	-	-	-	-
1-2	20 (overnight)	++	+++	+++	++	-	-	-
2	37	-	-	-	-	-	-	-
	20	++	+++	+	-	-	-	-
	37	-	-	-	-	-	-	-
	20	-	+++	++	-	-	-	-
	37	-	-	-	-	-	-	-

- = no agglutination;
± = doubtful positive;
+, ++, +++ = increasing degrees of agglutination.

Inhibition of the haemagglutinin

The *Cl. welchii* haemagglutinin was not inhibited by any of a wide range of carbohydrates in 1-2 per cent. solution; these included D-glucose, sucrose, maltose, dulcitol, mannitol, starch, D-mannose, D-glucosamine hydrochloride, and L-fucose. Aqueous extracts of the heated bacillary deposits of haemagglutinating and non-haemagglutinating strains of *Cl. welchii* contained no significant inhibitors of haemagglutination either by *Cl. welchii* haemagglutinin or by blood group-A antiserum.

Cooked-meat broth inhibitor C. The inhibition of the *Cl. welchii* haemagglutinin by sterile supernatant from cooked-meat broth was reported by Wickham (1956b), but she did not investigate the inhibitor involved and presumed that it was blood group-A substance derived from the peptone in the broth. Dilutions of cooked-meat broth supernatant up to 1 in 64 inhibited 1 unit or more of haemagglutinin (0.5 ml. amounts, see Methods) in the present study. The inhibitor content of sterile cooked-meat broth supernatant was not diminished by prolonged centrifugation at 3000 *r.p.m.*, by filtration through sintered glass or Seitz filters, by heat at 100°C. in the steamer for 1 hr or at 120°C. in the autoclave for 20 min. It was not dialysable through

cellophan against several changes of a large excess of distilled water at 4°C. for 2 days. Its activity was not altered in the presence of Tris-maleate buffers over the pH range 5.6 to 7.5 in haemagglutination-inhibition tests, nor was it destroyed on treatment of the diluted broth with trypsin, or with potassium periodate or carbon dioxide as performed in virus haemagglutination-inhibitor inactivations (Andrewes et al., 1959). A repeated attempt with potassium periodate confirmed that the inhibitor was not inactivated by this treatment.

The inhibitor could be precipitated by acetone. An equal volume of acetone was added to 11 ml. sterile cooked-meat broth supernatant at 20°C. and the resultant precipitate concentrated by centrifugation. The precipitate was then resuspended in 5 ml. saline and steamed to remove traces of acetone. The acetone-treated broth supernatant was freeze-dried and subsequently resuspended in 5 ml. distilled water. The inhibitory activities of the resuspended precipitate and the acetone-treated broth were then compared. Table 23 shows that the acetone precipitate retained much of the inhibitory activity which was virtually absent from the acetone-treated broth.

Table 23

The activity of *Cl. welchii* haemagglutinin (2 haemagglutinating doses) against human group-A red cells in the presence of an inhibitory substance present in cooked-meat broth (C.M.B.) supernate and precipitable by acetone.

Material tested	Haemagglutination observed in presence of material diluted 1 in							
	2	4	8	16	32	64	128	256
Untreated sterile C.M.B. supernate	-	-	-	-	-	-	±	±
Seitz-filtered C.M.B. supernate	-	-	-	-	-	-	±	+++
Acetone-treated C.M.B. supernate (concentrated x 5)	-	+++	+++	+++	+++	+++	+++	+++
Aqueous solution of Acetone-insoluble precipitate from C.M.B. supernate (concentrated x 2)	-	-	-	-	-	-	+++	+++

- = no agglutination (inhibitor present)

± = doubtful agglutination

+, ++ = increasing degrees of agglutination

+++ = complete agglutination (inhibitor absent)

The inhibitor was not readily absorbed by charcoal, normal red cells of man or sheep, or bacillary deposit of a haemagglutinating or non-haemagglutinating culture of *Cl. welchii* at 4°C.

When the cooked-meat broth inhibitor, hereafter designated "inhibitor C", was added to normal red cells which were then washed before exposure to haemagglutinin, the red cells remained agglutinable. This shows that the inhibitor does not become firmly bound to normal red cells. Red cells which have been agglutinated by *Cl. welchii* haemagglutinin and then dispersed and washed with saline, normally always re-agglutinate on settling at 20°C. When such *Cl. welchii*-agglutinated red cells were washed in saline and re-suspended in sterile broth from cooked-meat medium, they did not later re-agglutinate. When they were then washed free of inhibitor and suspended in fresh saline they agglutinated atypically or settled in apparently negative patterns. This may have been due to partial retention of inhibitor at the altered red cell surface.

Peptone inhibitor A. An active inhibitor, inhibitor A, of human group-A red cell isoagglutination by blood group-A antiserum occurs in peptone (Schiff, 1939). The peptone-containing supernatant of cooked-meat broth therefore inhibits blood group-A isoagglutination, and Wickham (1956b)

thought that the isoagglutination inhibitor (A) of peptone was the same as the haemagglutination inhibitor (C) of cooked-meat broth. The results of the present investigation suggest that the two inhibitors are not identical.

Differentiation between inhibitors A and C. Results of many experiments indicated that, although sterile cooked-meat broth supernatant strongly inhibited both the Cl. welchii haemagglutinin and the blood group-A isoagglutinin, a 1 per cent. solution of peptone itself inhibited the Cl. welchii haemagglutinin very slightly but inhibited the blood group-A isoagglutinin strongly. On the other hand, a simple saline extract of cooked-meat, obtained by autoclaving approximately 1 g. of the cooked-meat used for cooked-meat broth in 15 ml. of saline, contained a powerful inhibitor (C) of Cl. welchii haemagglutinin but little inhibitor (A) of the blood group-A isoagglutinin. For example, in a particular experiment final dilutions of the saline extract of cooked-meat from 1 in 6 to 1 in 24 completely inhibited 4 haemagglutinating units of Cl. welchii haemagglutinin, but these dilutions had no inhibitory effect on 4 haemagglutinating units of blood group-A antiserum. Similar dilutions of 1 per cent. peptone in saline did not inhibit Cl. welchii haemagglutinin, but, even at a dilution of 1 in 96,

the peptone solution strongly inhibited 4 haem-agglutinating units of blood group-A antiserum. A 1 per cent. solution of commercial meat extract ("Lab Lemco") lacked any inhibitory activity.

Substances known to inhibit haemagglutination by myxoviruses were found to inhibit the Cl.welchii haemagglutinin but they did not inhibit the blood group-A isoagglutinin. Table 24 summarises the results of experiments in which three haemagglutinating systems, Cl. welchii haemagglutinin, blood group-A antiserum and influenza virus PR8, were tested separately against human group-A red cells in the presence of several inhibitory substances. Saline extract of cooked meat strongly inhibited influenza virus, Newcastle disease virus and Cl. welchii haemagglutinins whereas the peptone solution inhibited them only very slightly. Egg-white mucin inhibited influenza virus and Cl. welchii haemagglutinin, but not the blood group-A isoagglutinin.

Table 24

The differential inhibitory effect of four substances tested in solution against three haemagglutinating systems employing human group-A red cells.

In the presence of	HAEMAGGLUTININ		
	Cl. welchii haem-agglutinin	blood group-A iso-agglutinin	influenza virus PR8
Saline	-	-	-
Cooked-meat broth supernate	III	III	III
1 per cent. peptone water	ɪ	III	ɪ
Saline extract of cooked meat	III	-	III
egg-white solution	III	-	III

- = no inhibitory activity

ɪ = minimal inhibitory activity

III = powerful inhibitory activity

A heated extract of human saliva (blood group-O) actively inhibited the *Cl. welchii* haemagglutinin but not the blood group-A isoagglutinin. Finally, a specimen of semi-purified blood group-A

substance obtained through the courtesy of Dr J. Koscielak at Dr W.T.J. Morgan's laboratory, the Lister Institute, London, was used to show specific inhibition of 4 haemagglutinating units of the blood-group-A isoagglutinin in dilutions of a 0.3 per cent. solution up to 1 in 128 of the blood-group-A active material, whereas there was no inhibition of 4 haemagglutinating units of Cl. welchii haemagglutinin even at a 1 in 2 dilution of the blood-group-A active solution.

Inhibitor destruction

Inhibitor C breakdown. When 2 ml. of crude filtrate of V. cholerae containing RDE was incubated at 37°C. for 20 hr with 10 ml. cooked-meat broth supernatant and the V. cholerae filtrate thereafter inactivated by heat, the inhibitor C content of the broth supernatant was destroyed. The broth's content of the peptone inhibitor A was not reduced by this treatment but there was reduction of its inhibitory effect against haemagglutination by Newcastle disease virus. Control tests, held at 4°C. during the incubation period and subsequently inactivated along with the others, showed no loss of either inhibitor A or C.

Cl. welchii was similarly capable of destroying inhibitor C. The centrifuged supernatant

fluids from cooked-meat broth cultures showed loss of inhibitor C during growth of the culture and this could be demonstrated in several ways. For instance, Table 25 shows the results of an experiment in which six tubes of pooled sterile cooked-meat broth supernatant were each inoculated with a standard loopful of a young cooked-meat broth culture of a different strain of *Cl. welchii.* After growth for 18 hr at 37°C., the cultures were centrifuged, the supernatants heated for 10 min. in the steamer to inactivate any haemagglutinin, and the inhibitor C content thereafter determined by adding graded doses of each heated supernatant to a series of tubes containing 16 haemagglutinating units of *Cl. welchii* haemagglutinin. The mixtures were shaken and allowed to stand on the bench for 1 hr before human group-O red cells were added. The occurrence of haemagglutination in any tube indicated that inhibitor had been destroyed. An uninoculated tube of cooked-meat broth supernatant was incubated and treated in parallel with the tests and showed full inhibitory activity.

Table 25

The activity of equal doses of Cl. welchii haemagglutinin in the presence of graded doses of heat-inactivated 18 hr culture supernatants of six different strains of Cl. welchii.

Culture supernate of strain	Amount of heated culture supernate added to test (0.02 ml. drops)								i.e. inhibitor destruction
	1	2	3	4	5	6	8	10	
A	+++	+++	+++	+++	+++	+++	+++	+++	complete
C	+++	+++	+++	+++	+++	+++	+++	+++	complete
E	+++	+++	+++	+++	+++	+++	+++	+++	complete
F	±	-	-	-	-	-	-	-	nil
W	+++	+++	+++	+	++	+	++	/	almost complete
443	+++	+++	+++	+++	+++	+++	+++	+++	complete
Uninoculated control	-	-	-	-	-	-	-	-	nil

- = no agglutination

± = doubtful positive

+, ++, +++ = increasing degrees of agglutination

/ = test lost

In another series of experiments the heated culture supernatants of 48 hr cooked-meat broth cultures of many strains of Cl. welchii were used as diluent for parallel serial doubling dilution

tests of a standard Cl. welchii haemagglutinin (Table 26). This was a sensitive method for detecting residual inhibitor when destruction of inhibitor C by these cultures was considerable but incomplete.

Table 26

Varying degrees of destruction of inhibitor C indicated by the activity of a standard Cl. welchii haemagglutinin diluted in the heat-inactivated 48 hr cooked-meat broth culture supernates of 13 strains of Cl. welchii.

DILUENT	Standard Cl. welchii haemagglutinin diluted 1 in					
Culture supernate of strain	4	8	16	32	64	128
443	+++	+++	+++	++	±	-
032	+++	+++	+++	++	±	-
W	+++	++	++	±	-	-
754	+++	++	+	±	-	-
C	+++	+++	+++	±	-	-
D	+++	+++	+	±	-	-
E	+++	+++	++	-	-	-
B	+++	++	+	-	-	-
A	+++	+++	±	-	-	-
153	+++	++	+	-	-	-
029	+++	++	+	-	-	-
F	++	++	±	-	-	-
Sterile cooked-meat broth supernate	++	±	-	-	-	-
Saline	+++	+++	+++	+++	±	-

Haemagglutination occurring between these limits is proportional to the degree of inhibitor destruction

- = no agglutination; ± = doubtful positive;

+, ++, +++ = increasing degrees of agglutination.

The bacterial deposit from 10 ml. glucose broth overnight cultures of most strains of _Cl. welchii_, irrespective of haemagglutinin production, was capable of destroying the inhibitor C in 5 ml. cooked-meat broth supernatant in 20 hr at 37°C. The bacterial deposits of strain F and two non-haemagglutinating food-poisoning strains (153 and 029) were relatively inactive. In a typical experiment (Table 27) the cooked-meat broth supernatant was centrifuged free of bacteria after incubation for 20 hr and was then inactivated by heating at 60°C. for 30 min. Thereafter, each treated supernatant was serially diluted in saline and a standard dose of 2 haemagglutinating units of _Cl. welchii_ haemagglutinin added to each tube. The mixtures were shaken and, after 15 min. at bench temperature, red cell suspension was added to each tube.

Destruction of inhibitor C by _Cl. welchii_ was also demonstrated using Newcastle disease virus as haemagglutinin. Table 28 shows the results of an experiment in which a laboratory stock strain before and after guinea-pig passage (A and Agp) was tested for ability to destroy inhibitor C. The two strains were cultured for 20 hr in nutrient broth and the cultures centrifuged. The bacterial deposits were washed once in saline and then resuspended in saline.

Table 27. Destruction of inhibitor C content of cooked-meat broth supernate by glucose broth culture deposits of eight strains of *Cl. welchii*.

Cooked-meat broth supernate treated with glucose broth culture deposit of strain	Haemagglutination produced by *Cl. welchii* haem-agglutinin added to treated cooked-meat broth supernate diluted 1 in:					
	2	4	8	16	32	64
A	-	+	+++	+++	+++	+++
C	+++	+++	+++	+++	+++	+++
443	+++	+++	+++	+++	+++	+++
032	+++	+++	+++	+++	+++	+++
754	-	+++	+++	+++	+++	+++
L2A	++	+++	++	+++	+++	+++
W	-	+	++	+++	+++	+++
153	-	-	-	-	-	-
Untreated cooked-meat broth supernate	-	-	-	-	-	-

+, ++, +++ = increasing degrees of haemagglutination, (i.e. decreasing degrees of inhibition).

- = complete inhibition of haemagglutinin.

Table 28

Haemagglutinating activity of equivalent doses of Newcastle disease virus diluted in treated cooked-meat broth supernates.

Diluent		Virus suspension diluted 1 in							i.e. inhibitor
		4	8	16	32	64	128	256	
Sterile saline	Test at 4°C.	+++	+++	+++	+++	+++	++	-	absent
	Test at 37°C.	+++	+++	+++	+++	+++	++	-	absent
CMB supernate treated for 18 hr		Virus suspension diluted 1 in							i.e. inhibitor
with	at °C.	4	8	16	32	64	128	256	
Sterile saline	4	+	±	-	-	-	-	-	present
	37	+	±	-	-	-	-	-	present
Sterile nutrient broth	4	+	±	-	-	-	-	-	present
	37	±	±	-	-	-	-	-	present
Bact. deposit of strain A	4	±	-	-	-	-	-	-	present
	37	+++	+++	+++	+++	+++	+	-	inactivated
Cult. supernate of strain A	4	++	+	±	-	-	-	-	present
	37	++	++	+	±	±	-	-	slightly inactivated
Bact. deposit of strain A gp	4	+	±	-	-	-	-	-	present
	37	+++	+++	+++	+++	+++	++	-	inactivated
Cult. supernate of strain A gp	4	+	±	-	-	-	-	-	present
	37	+	+	±	+	±	-	-	slightly inactivated

- = no agglutination

± = doubtful positive

+, ++, +++ = increasing degrees of agglutination

Aliquots (1 ml.) of sterile pooled cooked-meat broth supernatant were mixed either with culture supernate or resuspended bacterial deposit and the mixtures were incubated aerobically at 37°C. for 18 hr. A duplicate series of tests was held meanwhile at 4°C. and tubes containing mixtures of pooled cooked-meat broth supernatant and saline only, were included at both temperatures as controls. After incubation, 0.2 ml. amounts of the treated broth supernatants were used as diluent in parallel titrations of a standard dose of Newcastle disease virus. Virtually complete destruction of inhibitor C by the bacterial deposits was demonstrated; the inhibitor C-destroying activity of the passaged strain was not reduced and appeared to be slightly enhanced. The culture supernates were much less active. There was no inhibitor C destruction in the tests held at 4°C., suggesting that inhibitor C destruction is enzymatic.

<u>Inhibitor A destruction</u>. The ability of a strain to destroy inhibitor A was assessed by incubating the bacterial deposit of an overnight glucose broth culture with 5 ml. 1 per cent. peptone at 37°C. and thereafter determining the residual inhibitor A content by isoagglutination-inhibition tests. Another method of demonstrating inhibitor A destruction was to grow cultures for 48 hr in 1 per cent. peptone water and thereafter assess the inhibitor A content of the centrifuged culture supernates. It was important to note that cultures of haemagglutinating strains of <u>Cl. welchii</u> render red cells panagglutinable and that the technique employed by Wickham (1956b) to demonstrate destruction of blood group-A substance (inhibitor A) did not appear to allow for the possibility that induction of panagglutinability rather than destruction of the A inhibitor gave the positive results in her tests. In the present work it was found that the agent inducing panagglutinability associated with the haemagglutinin is easily inactivated by heat; broth cultures were therefore heated and centrifuged free of precipitate before testing for their inhibitor content.

Table 29 shows the inhibitor A activity of heat-inactivated supernatants of 48 hr cultures of 12 strains of <u>Cl. welchii</u>. In this experiment,

each culture supernatant was used as diluent for parallel titrations of blood group-A antiserum so that small amounts of residual inhibitor could be detected. The occurrence of agglutination indicated destruction of inhibitor.

Table 29

Agglutination of human group-A red cells by blood group-A antiserum in the presence of heat-inactivated peptone water culture supernates.

Diluent	blood group-A antiserum diluted 1 in					i.e. inhibitor A
	4	8	16	32	64	
Saline	+++	+++	+++	+++	+	absent
Sterile 1 per cent. peptone water	-	-	-	-	-	present
Sterile 1 per cent. peptone water after incubation and heating	-	-	-	-	-	present
Heat-inactivated 48 hr peptone water culture supernate of strain						
A	+++	+++	-	-	-	reduced
B	+++	++	+	-	-	reduced
C	+++	++	-	-	-	reduced
D	+++	+++	++	+	-	reduced
E	+++	+	-	-	-	reduced
F	±	-	-	-	-	present
W	+++	+++	+	-	-	reduced
443	++	-	-	-	-	slightly reduced
153	±	-	-	-	-	present
029	-	-	-	-	-	present
032	-	-	-	-	-	present
754	-	-	-	-	-	present

The ability of a strain to destroy inhibitor C was unrelated to its ability to destroy inhibitor A and it was also unrelated to its current actual or potential haemagglutinin production. Table 30 summarises representative results of many experiments in which haemagglutinating and non-haemagglutinating strains of Cl. welchii were examined for their ability to destroy inhibitors A and C. The ability of different strains to destroy inhibitor A seemed to be related to their capacity for production of haemagglutinin, but these activities did not appear to be due to the same factor because they were not always encountered together in the same culture medium. Glucose broth cultures of haemagglutinating strains yielded non-haemagglutinating bacterial deposit which was active in destroying inhibitor A and, similarly, peptone water cultures were non-haemagglutinating although the inhibitor A content of the medium was destroyed during growth.

Table 30

Haemagglutinating (HA), inhibitor C destroying (CD) and blood group substance A destroying (AD) activities of eight strains of Cl. welchii.

Strain of Cl. welchii	Activity		
	HA	CD	AD
A	+++	++	+++
L2A	+++	+++	++
W	+++	+	++
C	+	+++	+
443	+	+++	+
F	-	±	-
153	-	-	-
032	-	+++	±

- = no activity

± = doubtful positive

+, ++, +++ = increasing degrees of activity

Inhibitor C destruction as indicated by loss of inhibitor of *Cl. welchii* haemagglutinin and virus haemagglutinin was compared simultaneously with inhibitor A destruction in the following experiment. Bacterial deposits from 10 ml. amounts of 20 hr glucose broth cultures were each resuspended in 5 ml. sterile pooled cooked-meat broth supernate and the mixtures incubated aerobically at 37°C. for 20 hr. Tubes of uninoculated cooked-meat broth supernate and tubes of broth supernate containing 1 ml. of *V. cholerae* filtrate were held as controls at 4°C. and 37°C. during this period. The mixtures were thereafter held at 60°C. for 30 min. to inactivate any contained haemagglutinin and three sets of serial dilutions of each were then prepared in saline. Standard doses (1 - 2 haemagglutinating units) of Newcastle disease virus, *Cl. welchii* haemagglutinin or blood group-A antiserum were added to each of the three sets of tests and thoroughly mixed. After 15 min. at bench temperature, 0.5 ml. aliquots of 1 per cent. group-A red cells were added and, after shaking, the tests were allowed to settle. The results indicated the degree of destruction of inhibitors C and A in each case and this was compared with the known potential haemagglutinin production of each strain tested (Table 31). These results

Table 31

The activity of three different haemagglutinating systems in the presence of serial dilutions of treated cooked-meat broth (CMB) supernates, and the potential haemagglutinin (HA) production of each strain of *Cl. welchii* employed.

Substance tested		HAEMAGGLUTININ																	Potential HA activity
Heated CMB supernate after treatment with		Newcastle disease virus						Cl. welchii haemagglutinin						blood group-A antiserum					
		Standard dose suspended in CMB supernate diluted 1 in																	
		2	4	8	16	32	64	2	4	8	16	32	64	2	4	8	16	32	
Cl. welchii at 37°C.	A	+++	+++	+++	+++	+++	+++	-	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	C	±	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	++	+++	+++	+++	+
	443	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	++	++	+++	±
	032	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+	++	++	+++	-
	754	+++	+++	+++	+++	+++	+++	-	+++	+++	+++	+++	+++	+	++	++	+++	++	-
	L2A	-	+++	+++	+++	+++	+++	++	+++	++	+++	+++	+++	++	++	+++	+++	+++	+++
	W	±	+++	+++	+++	+++	+++	-	+	++	+++	+++	+++	++	++	+++	+++	+++	+++
	153	-	-	-	-	-	-	-	-	-	-	-	-	-	+	++	++	+++	-
V. cholerae filtrate at 4°C.		-	-	-	-	-	±	-	-	-	-	-	-	-	-	++	++	+++	
V. cholerae filtrate at 37°C.		-	-	-	±	+++	+++	++	+++	+++	+++	+++	+++	-	-	++	++	+++	
Untreated CMB supernate		-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	++	+++	

- = no activity; ± = doubtful positive;

+, ++, +++ = increasing degrees of activity.

are consistent with the observations previously made concerning the relationship between inhibitor C destruction, inhibitor A destruction and haemagglutinin production by _Cl. welchii_ cultures. In addition, there is an indication that the inhibition of the _Cl. welchii_ haemagglutinin does not entirely parallel that of the virus haemagglutinin although the two appear to be closely related. Thus, although inhibitor C may represent an inhibitor complex with slightly different affinities for the two haemagglutinins, on the present available evidence it cannot be regarded as a single substance. Inhibitor C is presumably a substance of animal origin containing neuraminic acid in bound form. The presence of neuraminic acid residues in cooked-meat broth has been confirmed in the present work (see p. 184) and further investigation of this interesting inhibitor is indicated.

Demonstration of apparent RDE activity

Initial attempts to demonstrate RDE activity associated with haemagglutinating activity in *Cl. welchii* cultures met with many difficulties. Well-defined results such as those recorded by Wickham (1956b) in *Cl. welchii* RDE experiments were not readily reproduced with haemagglutinating strains. Haemolysis was especially troublesome with freshly isolated strains in these tests. When cooked-meat broth cultures were used, the possible presence of inhibitor C, which had been shown to inhibit myxovirus haemagglutinin as well as *Cl. welchii* haemagglutinin, added a further complication. Nevertheless it was possible to demonstrate an RDE effect by treatment of red cells with cooked-meat broth culture supernates; Table 32 shows the haemagglutinating activity of some 4-day cooked-meat broth culture supernates, and Table 33 shows the RDE effect demonstrable when 0.2 ml. amounts of the same supernates were serially diluted in saline containing 1 per cent. sodium citrate and incubated at 37°C. for 4 hr with 0.2 ml. aliquots of a 1 per cent. suspension of human group-O red cells. Thereafter, the supernatant fluids were pipetted from the deposited red cells which were then resuspended in 0.4 ml. aliquots of saline containing ten haemagglutinating units of influenza virus PR8. Haemagglutination

Table 32

The haemagglutinating activity of centrifuged supernatants of 4-day cooked-meat broth cultures of twelve strains of *Cl. welchii* tested against human group-O red cells.

Strain	Culture supernatant diluted 1 in							
	2	4	8	16	32	64	128	256
A	+++	+++	+++	++	+	-	-	-
B	+++	+++	+++	++	+	-	-	-
C	1+++	+	-	-	-	-	-	-
D	+++	+++	++	±	-	-	-	-
E	++	++	±	-	-	-	-	-
F	±	-	-	-	-	-	-	-
W	+++	+++	+++	+++	++	++	+	-
029	-	-	-	-	-	-	-	-
754	-	-	-	-	-	-	-	-
032	-	-	-	-	-	-	-	-
153	++	-	-	-	-	-	-	-
443	++	±	-	-	-	-	-	-
Uninoculated control	-	-	-	-	-	-	-	-

Table 33

The RDE effect produced by 4-day cooked-meat broth culture supernates of twelve strains of *Cl. welchii* after incubation for 4 hr at 37°C. with human group-O red cells (1% sodium citrate in saline diluent) to which 10 HA units of influenza PR8 virus were subsequently added.

Strain	Red cells treated with culture supernate diluted 1 in						
	4	8	16	32	64	128	256
A	(+++)	(+++)	(+++)	+++	+++	+++	+++
B	(+++)	(+++)	(-)	-	+++	+++	+++
C	+++	±	-	+++	+++	+++	+++
D	(+++)	(+++)	-	+++	+++	+++	+++
E	(+++)	-	-	+++	+++	+++	+++
F	+++	+++	+++	+++	+++	+++	+++
W	(+++)	(++)	(++)	(++)	+++	+++	+++
029	+++	+++	+++	+++	+++	+++	+++
754	-	+++	+++	+++	+++	+++	+++
032	+++	+++	+++	+++	+++	+++	+++
153	-	+++	+++	+++	+++	+++	+++
443	L	L	L	L	L	L	-
Uninoculated control	+++	+++	+++	+++	+++	+++	+++

- = no agglutination, i.e. RDE effect present.
± = doubtful agglutination
++,+++ = increasing degrees of haemagglutination
L = complete lysis
() = indicate possible extent of residual *Cl. welchii* haemagglutinin activity.

patterns were read after 2 hr at bench temperature.

In some cases, persisting haemagglutination due to Cl. welchii haemagglutinin masked any RDE effect, e.g. strains A and W (Table 33), but in many cases the RDE effect was produced at one or two dilutions beyond the haemagglutinin titre. An RDE effect was produced by some cultures of non-haemagglutinating strains, but haemolysis was frequently marked in tests of these cultures. When RDE tests were performed as previously described but held overnight at bench temperature instead of incubating for 4 hr at 37°C., haemolysis was less troublesome and an apparent RDE effect was very much more evident (Table 34). In all such tests a tube of sterile uninoculated cooked-meat broth was included as a control to ensure that inhibitor C did not remain to produce false negative patterns.

Haemolysis was also suppressed, andan apparent RDE effect was more evident, when dilutions of 48 hr cooked-meat broth culture supernates were mixed with aliquots of human group-O red cells suspended (1 per cent. v/v) in saline containing 2 per cent. sodium citrate. These mixtures were incubated for 3 hr at 37°C. and the supernatant fluids thereafter removed from the deposited red cells which were resuspended in 2 per cent.sodium

Table 34

The RDE effect produced by 4-day cooked-meat broth culture supernates of twelve strains of Cl. welchii after overnight contact at room temperature with human group-O red cells (saline diluent) to which, after washing, 10 HA units of influenza virus PR8 were subsequently added.

Strain	Red cells treated with culture supernate diluted 1 in							
	2	4	8	16	32	64	128	256
A	(+++)	(+++)	(+++)	(+++)	(+++)	(+)	-	-
B	(+++)	(+++)	(+++)	(+++)	(+++)	(±)	-	-
C	(L)	(±)	-	-	-	++	+++	+++
D	(L)	(+++)	(+++)	(++)	-	-	+++	+++
E	(L)	(+)	-	-	±	±	±	+++
F	+	+++	+++	+++	+++	+++	+++	+++
W	(L)	(+++)	(+++)	(+++)	(+++)	(+++)	(+++)	+++
029	+++	+++	+++	+++	+++	+++	+++	+++
754	-	-	-	+++	+++	+++	+++	+++
032	(L)	-	-	+++	+++	+++	+++	+++
153	(±)	-	-	-	±	+++	+++	+++
443	(L)	-	-	-	++	+++	+++	+++
Uninoculated control	+++	+++	+++	+++	+++	+++	+++	+++

- = no agglutination

± = doubtful agglutination

+, ++, +++ = increasing degrees of haemagglutination

L = complete lysis

() indicate possible extent of residual Cl. welchii haemagglutinin activity.

citrate in saline and held at 4°C. overnight. The supernatant fluid was then removed and fresh citrate-saline added to the deposited cells which were again allowed to settle at 4°C. Finally, the supernatant fluid was removed and the red cells resuspended in 0.8 ml. aliquots of saline containing 10 haemagglutinating units of Newcastle disease virus (Table 35). Cl. welchii-agglutinated red cells prepared in this way did not remain agglutinable and showed apparently well-defined RDE effects with cultures of non-haemagglutinating as well as haemagglutinating strains; control tests of the heat-inactivated culture supernates used in this experiment showed that in most cases inhibitor C had been destroyed, and in all cases inhibitor C had been removed by the changes of suspending fluid prior to the addition of virus.

The tests described, which demonstrated an apparent RDE effect, were developed from similar tests which are in routine laboratory use. These tests are open to the charge, however, that an RDE effect could be produced by any substance which blocks access to the myxovirus receptor sites at the red cell surface; as this does not necessarily involve receptor destruction, it is incorrect to consider that the above evidence confirms the presence of a receptor-destroying

Table 35

RDE effect produced by supernate of 48 hr cooked-meat broth cultures of 12 strains of *Cl. welchii* when incubated for 3 hr at 37°C. with group O red cells in the presence of sodium citrate (see text).

Strain	Reciprocal of haemagglutinin titre	Red cells treated with culture supernate diluted 1 in					
		4	8	16	32	64	128
A	32	-	-	-	-	++	+++
B	32	-	-	-	-	-	+++
C	4	-	-	-	-	+	+++
D	2	-	-	-	-	+++	+++
E	2	-	-	-	-	+++	+++
F	-	+	+++	+++	+++	+++	+++
W	32	-	-	-	-	-	+++
443	4	-	-	-	-	+++	+++
029	-	-	-	-	-	+++	+++
153	2	-	-	-	++	+++	+++
032	-	-	-	-	-	+++	+++
754	-	-	-	-	-	+++	+++
Uninoculated cooked-meat broth supernate		+++	+++	+++			

- = no activityin presence of Newcastle disease virus.

+, ++, +++ = increasing degrees of activity

enzyme in the cultures which produced an apparent RDE effect. For this reason, the mechanism of the RDE effect was further studied.

Effect of temperature on apparent RDE effect.

The RDE effects produced by culture supernates of 21 hr cooked-meat broth cultures of strain A before and after guinea-pig passage were tested at 20°C. and at 4°C. Human group-A red cells were exposed to dilutions of the culture supernates for 2 hr at the respective temperatures and, after washing once with saline, were resuspended in saline containing ten haemagglutinating units of Newcastle disease wirus. All of the reagents used in the tests at 4°C. were held at that temperature before starting the experiment. Although a parallel haemagglutination-inhibition test using NDV showed inhibitor C destruction to be more complete in culture supernate of Agp than in that of A, the RDE effect produced by A was greater than that with Agp. The results of the RDE tests (Table 36) showed that the apparent RDE effect was slightly but not significantly influenced by the different temperatures. This unexpected finding was investigated in detail using a 4-day blood agar culture of strain A harvested in 8 ml. sodium citrate buffer at pH 5.6. Dilutions were made in the citrate buffer and parallel tests of haemagglutinating and "RDE"

Table 36

The RDE effect produced by treatment of human group-A red cells with 21-hr cooked-meat broth culture supernates in 2 hr at 20°C. and 4°C.

Temperature	Red cells treated with culture supernate						
	of strain	diluted 1 in					
		2	4	8	16	32	64
20°C.	A	-	-	-	-	-	±
	Agp	-	-	-	±	+++	+++
4°C.	A	-	-	-	-	±	+
	Agp	-	-	++	+++	+++	+++

(Ten haemagglutinating doses of Newcastle disease virus added.)

+, ++, +++ = increasing degrees of haemagglutination.

± = doubtful positive.

- = no agglutination, (i.e. RDE effect produced).

activity done on centrifuged supernate, resuspended bacterial deposit and ultrasonically disintegrated bacterial deposit at various temperatures, using an extended incubation time of 18 hr. The results obtained with the disintegrated material are representative and show that the receptor-inactivating effect for NDV was not temperature dependent and was not confined to the limits of the haemagglutinin's activity (Table 37).
These observations were fully confirmed by the results of later experiments (q.v.). Washings from an uninoculated anaerobically-incubated blood agar plate caused no receptor-blocking effect.

Table 37

The receptor-inactivating activities of disintegrated bacterial deposit harvested from a 4-day blood agar culture of strain A in citrate buffer at pH 5.6 and tested against human group-A red cells at different temperatures. (Receptor-inactivating readings after exposure for 18 hr at the stated temperature). - Newcastle disease virus subsequently added as haemagglutinin.

Temp. of exposure for 18 hr	Resuspended disintegrated bacterial deposit at a dilution of 1 in									
	4	8	16	32	64	128	256	512	1024	2048
4°C.	(+++)	(+++)	(+++)	(+++)	(++)	-	-	+++	+++	+++
20°C.	(+++)	(+++)	(++)	(±)	-	-	-	-	+++	+++

- = no haemagglutination

± = doubtful positive

+, ++, +++ = increasing degrees of haemagglutination

() indicate HA attributable to Cl. welchii haemagglutinin activity tested at the appropriate temperature.

<u>Effect of pH on receptor-inactivating effect.</u> The receptor-blocking effect was particularly sensitive to pH and this was apparent at room temperature or in the cold, but it was less evident in tests performed at 37°C. Table 38 shows the haemagglutinating activity of the centrifuged supernate of a 48 hr blood agar culture tested at different pH values against human group-A red cells (citrate-phosphate buffers). The appropriate buffer was used as diluent in these tests and as suspending agent for the red cells used in each test. When the red cells had settled and haemagglutination patterns had been noted after 2 hr at bench temperature, the supernatants in each tube were discarded and aliquots of saline containing 10 haemagglutinating units of Newcastle disease virus were added. After shaking the tests to resuspend the red cells, these were left in turn for a further 2 hr to settle at bench temperature. The results, (Table 39), show a well-defined receptor-blocking effect at pH values from 5.4 to 6.4. Very similar patterns were produced in a duplicate series of tests in which the dilutions of culture supernate were prepared and red cells added at bench temperature but immediately thereafter centrifuged out of the mixtures in a refrigerated centrifuge*. The supernates were discarded and 10

* see Table 40.

Table 38

The haemagglutinating activity of 48 hr culture supernate of strain A at different pH values against human group-A red cells.

pH of test	Culture supernate diluted 1 in								
	4	8	16	32	64	128	256	512	1024
5.4	+++	±	±	-	-	-	-	-	-
5.6	+++	+++	++	+	±	-	-	-	-
5.8	+++	+++	±	-	-	-	-	-	-
6.4	+++	+++	++	±	-	-	-	-	-
6.8	+++	+++	+++	±	-	-	-	-	-
7.2	+++	++	-	-	-	-	-	-	-
7.7	+++	+++	-	-	-	-	-	-	-
Saline control	+++	+++	+++	-	-	-	-	-	-

- = no activity

± = doubtful positive

+, ++, +++ = increasing degrees of activity

Table 39

Receptor-inactivation produced by dilutions of 48 hr culture supernate of strain A after contact for 2 hr at 20°C. with human group-A red cells. (Newcastle disease virus used as haemagglutinin).

at pH	Red cells treated with culture supernate diluted 1 in								
	4	8	16	32	64	128	256	512	1024
5.4	(++)	-	-	-	+++	+++	+++	+++	+++
5.6	-	(±)	-	-	+++	+++	+++	+++	+++
5.8	-	-	-	±	+++	+++	+++	+++	+++
6.4	-	-	-	-	++	+++	+++	+++	+++
6.8	(+++)	(+++)	(+)	+	+++	+++	+++	+++	+++
7.2	(+)	(+)	-	++	+++	+++	+++	+++	
7.7	(+++)	(±)	-	+	+++	+++	+++	+++	

\- = no haemagglutination

± = doubtful positive

+, ++, +++ = increasing degrees of haemagglutination.

() indicate HA attributable to Cl. welchii haemagglutinin activity tested at the appropriate temperature.

Table 40

Receptor-inactivation produced by dilutions of 48 hr culture supernate of strain A after transient exposure to red cells at 20°C. (see text). (Newcastle disease virus used as haemagglutinin).

at pH	Red cells treated with culture supernate diluted 1 in								
	4	8	16	32	64	128	256	512	1024
5.4	-	-	+++	+++	+++	+++	+++	+++	+++
5.6	-	-	++	+++	+++	+++	+++	+++	+++
5.8	-	-	-	+++	+++	+++	+++	+++	+++
6.4	-	-	-	+++	+++	+++	+++	+++	+++
6.8	(+++)	(+++)	(++)	+++	+++	+++	+++	+++	+++
7.2	(+++)	(±)	±	+++	+++	+++	+++	+++	+++
7.7	(+++)	(±)	±	+++	+++	+++	+++	+++	+++

- = no haemagglutination

± = doubtful positive

+, ++, +++ = increasing degrees of haemagglutination

() indicate HA attributable to *Cl. welchii* haemagglutinin activity tested at the appropriate temperature.

haemagglutinating units of Newcastle disease virus added to each tube as above, the tests being allowed to settle at room temperature. The similar patterns observed in these tests to those of the tests held for some time at bench temperature, suggest that a relatively temperature-independent adsorption mechanism may account for some of the receptor blocking effect. In a third series, the culture supernate-red cell mixtures were held at 37°C. for 2 hr before the supernates were discarded and the Newcastle disease virus added. These tests were allowed to settle thereafter at room temperature. The results (Table 41) were much less well-defined and initially indicated receptor inactivation maximal at pH 7.2. When these tests were shaken and read after a further 2 hr at bench temperature, there appeared to be a maximal effect in the pH range 5.6 - 6.4. Interpretation of these results is difficult; they illustrate the disadvantages of the red cell as an indicator in the presence of several substances which may be inter-acting simultaneously. McCrea (1947) pointed out that an assessment of RDE activity under these conditions must take into account the presence of lecithinase which may compete for a related receptor site. Thus, conditions which are best for the demonstration of RDE may be those which are least conducive

Table 41

Receptor-inactivation produced by dilutions of 48 hr culture supernate of strain A after contact for 2 hr at 37°C. with human group-A red cells at different pH values.

(Newcastle disease virus used as haemagglutinin).

at pH	Red cells treated with culture supernate diluted 1 in								
	4	8	16	32	64	128	256	512	1024
5.4		-	++	+++	+++	+++	+++	+++	+++
5.6	-	-	+	++	+++	+++	+++	+++	+++
5.8	-	-	++	+	+++	+++	+++	+++	+++
6.4	-	-	-	+	++	++	+++	+++	+++
6.8	-	-	-	++	+++	+++	+++	+++	+++
7.2	-	-	-	-	±	++	+++	+++	+++
7.7	-	-	±	+	++	++	+++	+++	+++

- = no activity

± = doubtful positive

+, ++, +++ = increasing degrees of activity

to lecithinase activity. The possibility that some of the haemagglutinin patterns are due to residual Cl. welchii haemagglutinin activity, whereas other positive haemagglutinations indicate failure of receptor inactivation, renders any interpretation of these results speculative, especially as elution of the Cl. welchii haemagglutinin and the indicator virus may also occur. In the absence of any clear indication as to the nature of the agent causing the apparent RDE effect this is provisionally referred to as receptor-inactivating substance.

It was now evident that sensitivity to pH was more likely to influence the results of 'RDE' tests than was temperature. This was confirmed by the following experiment. A 20 hr blood agar culture of strain 906 was harvested and diluted serially in physiological saline. Three series of dilutions were made and these were held at 4°C., 20°C. and 37°C. respectively for 1 hr. Aliquots of 1 per cent. human group-A red cell suspension in saline, at the appropriate temperature, were then added and, after shaking, the tubes were held at these temperatures for a further 2 hr when haemagglutination was found to be absent from all tests. The supernates were then removed from the deposited red cells and 5 haemagglutinating units of Newcastle disease virus added to each tube, at the appropriate temperature.

Table 42 shows that the receptor-inactivation was minimal and that the effect was not temperature-dependent. When the same experiment was repeated using citrate-phosphate buffer at pH 6 as the suspending agent for the culture, a much clearer receptor-inactivating effect was evident, but again this was not influenced by the temperature of the tests. A haemagglutinating strain, L2A, tested in parallel in this latter experiment, produced similar results complicated by the appearance in the initial dilutions of haemagglutination patterns attributable to the residual effect of the Cl. welchii haemagglutinin produced by this strain.

These results indicated that the receptor-inactivation effect was not due to the haemagglutinin, but it could be argued that a receptor-inactivation effect was a more sensitive manifestation of the presence of the haemagglutinin and could be demonstrated at dilutions beyond the haemagglutinin effect. To investigate this possibility, the following experiments were performed.

When older (48 hr) blood agar cultures were tested, a good receptor-inactivation effect could be obtained with saline alone as the diluent and harvesting fluid. Table 43 shows the haemagglutinating activity and receptor-inactivating activity of culture supernates from 48 hr blood

Table 42

Receptor-inactivation produced by treatment of human group-A red cells for 1 hr at different temperatures by cultures harvested in saline and in citrate phosphate buffer.

(Newcastle disease virus used as haemagglutinin).

Culture harvested in	Temp. of test	Strain	Culture diluted 1 in						
			4	8	16	32	64	128	256
Saline	4°C.	906	-	±	++	+++	+++	+++	/
	20°C.	906	-	±	++	+++	+++	+++	/
	37°C.	906	-	+++	+++	+++	+++	+++	/
M citrate-phosphate buffer pH 6	4°C.	906	-	-	-	+++	+++	+++	+++
	20°C.	906	-	-	-	+++	+++	+++	+++
	37°C.	906	-	-	+	+++	+++	+++	+++
	4°C.	L2A	(+++)	(+++)	(+++)	-	-	-	+++
	20°C.	L2A	(+++)	(+++)	(++)	-	-	-	+++
	37°C.	L2A	(+)	(±)	-	-	-	±	+++

- = no haemagglutination

± = doubtful positive

+, ++, +++ = increasing degrees of haemagglutination

() indicate haemagglutination attributable to *Cl. welchii* haemagglutinin.

/ = test not done

Table 43

The haemagglutinating and receptor-inactivating activities of supernates obtained from 48 hr blood agar cultures of three strains of Cl. welchii tested at different temperatures against human group-A red cells for 2 hr.

Temp. of test	Strain	HAEMAGGLUTINATION produced by culture supernate at a dilution of 1 in								RECEPTOR-INACTIVATION produced by culture supernate at a dilution of 1 in							
		2	4	8	16	32	64	128	256	2	4	8	16	32	64	128	256
4°C.	485	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	+++	+++
	B	+++	+++	+++	+++	-	-	-	-	±	+++	+++	+	-	-	+++	+++
	L2A	+++	+++	+++	+++	±	-	-	-	+++	+++	+++	++	±	+++	+++	+++
20°C.	485	-	-	-	-	-	-	-	-	-	-	-	-	-	±	+++	+++
	B	+++	+++	+++	±	-	-	-	-	(±)	(±)	-	-	-	-	-	+++
	L2A	+++	+++	+++	+++	-	-	-	-	(±)	(±)	(+++)	(++)	-	-	++	+++
37°C.	485	L	L	l	-	-	-	-	-	L	L	l	-	-	+	+++	+++
	B	+++	++	-	-	-	-	-	-	(±)	(±)	-	-	-	+	-	+
	L2A	L	L	+++	±	-	-	-	-	L	L	(±)	(±)	-	-	+	-

- = no haemagglutination; ± = doubtful positive;
+, ++, +++ = increasing degrees of haemagglutination;
() indicate haemagglutination attributable to Cl. welchii haemagglutinin.

agar cultures harvested in saline and tested against 1 per cent. suspension of human group-A red cells in saline, using saline as diluent, at 4°C., 20°C. and 37°C. In each case, the haemagglutination tests were read after standing at the indicated temperature for 2 hr. The saline dilutions of the supernates tested at 4°C. were held in the refrigerator for 30 min. before chilled suspensions of red cells at 4°C. were added. After the readings were noted at 2 hr, the supernates in each tube were discarded and the deposited red cells resuspended in aliquots of saline containing 5 haemagglutinating units of Newcastle disease virus. The tests were shaken and allowed to settle. The 37°C. and 20°C. tests were both held at 20°C. during this part of the experiment. The 4°C. tests were held <u>throughout</u> at 4°C. and chilled virus suspension was added. The non-haemagglutinating strain 485 showed a receptor-inactivating effect which was clearly not influenced by temperature. With strains B and L2A there was a significant receptor-inactivating effect demonstrable in the tests at 4°C., but this was slightly enhanced at 20°C. and significantly enhanced at 37°C. The results of this experiment were not replicable when the supernate of a 20 hr glucose broth culture of strain L2A was used. Excessive haemolysis invalidated tests

of the glucose broth culture at 37°C. but tests at 20°C. and 4°C. showed no receptor-inactivation. The blood agar supernates used in the previous experiment were treated with the deposited washed red cells from 10 ml. of a 1 per cent. suspension of group-A red cells by mixing and centrifuging the mixtures at 20°C.; the procedure took 20 min. The absorbed supernates were then tested for haemagglutinating and receptor-inactivating activity at room temperature. Table 44 shows that, after absorption by this method, the haemagglutinin was removed completely from culture supernate B and almost completely removed from culture supernate L2A. Nevertheless, the receptor-inactivating activity was slightly enhanced in all cases. This indicates that there is a temperature-independent receptor-inactivating substance in cultures of Cl. welchii which may simulate a receptor-destroying effect; the receptor-inactivating substance was best demonstrated in the presence of citrate and was present in older cultures. It appears to be different from the haemagglutinin and occurs in cultures of haemagglutinating and non-haemagglutinating strains.

Several difficulties and possible sources of error in the interpretation of tests which employ red cells as indicators of RDE (neuraminidase)

Table 44

Haemagglutinating and receptor-inactivating activities of *Cl. welchii* culture supernates against human group-A red cells before and after absorption with human group-A red cells.

Strain	haemagglutinin titre (20°C.)		receptor-inactivation titre (20°C.)	
	before absorption	after absorption	before absorption	after absorption
485	NIL	NIL	64	128
B	8	NIL	128	>256
L2A	16	2	64	64

activity had now been demonstrated. The aims of the work reported in the following section were therefore (i) to develop a more reliable biochemical test for neuraminidase activity and (ii) to perform enzyme assays on semi-purified fractions of _Cl. welchii_ cultures so that the relationship of neuraminidase to haemagglutinin in _Cl. welchii_ cultures could be defined.

The biochemical determination of neuraminidase activity in cultures of *Clostridium welchii*

Substrates for neuraminidase assay

1. Bovine submaxillary mucoprotein. The isolation from sheep submaxillary glands of an electrophoretically homogeneous mucoprotein which inhibited influenza virus haemagglutinin was reported by McCrea (1953). A similar inhibitory mucoprotein was isolated from bovine submaxillary glands by Curtain and Pye (1955). Using the methods developed by the latter workers, mucoprotain containing neuraminic acid was extracted from bovine submaxillary glands (see Methods). The product was excellent substrate for the development and assessment of neuraminidase assay techniques, but the yield of purified substrate obtained after treatment of the submaxillary gland tissue dissected from two cows was small (20 - 50 ml. of substrate solution); bovine submaxillary mucoprotein was therefore regarded as expensive in terms of time and labour and unlikely to be available for multiple tests. The mucoprotein was extracted from the glands of six cows in all so that preliminary development of the tests could continue while a search was made for a more readily available substrate material.

2. Egg-white. Chicken egg-white contains

several different mucoproteins of which two have been reported to contain neuraminic acid complexes (Werner and Odin, 1952; Gottschalk, 1956; Rhodes, Bennett and Feeney, 1960). The work of Feeney, Anderson, Azari, Bennett and Rhodes (1960) indicated that the neuraminic acid in egg-white is in the N-acetyl form; this all occurs bound to protein and can be liberated by Cl. welchii neuraminidase. Thus, egg-white promised to provide a readily available source of substrate for neuraminidase studies. Egg-white was separated from fresh eggs and homogenised with phosphate buffer under conditions which minimised frothing (see Methods). The blended egg-white was stored frozen and was used in most of the routine neuraminidase assays.

Determination of neuraminic acid content of substrates.

1. Bovine submaxillary mucoprotein. A 0.1 ml. amount of substrate solution was added to 0.9 ml. water and mixed with 1 ml. Bial's orcinol reagent. The absorption spectrum of the orcinol reaction products extracted in amyl alcohol (see Methods) is illustrated in fig. 2.

When the protein-bound neuraminic acid of 0.25 ml. bovine submaxillary mucoprotein was liberated by incubation with 0.5 ml. of an Ellner culture supernate of Cl. welchii strain L2A, the

absorption spectrum of the product in Warren's thiobarbituric acid assay showed maximal extinctions at about 550 mμ (fig. 2). The absorptions recorded in the range 500 - 600 mμ for the Bial orcinol and Warren thiobarbituric acid assays of these products are very similar to those obtained when 99 per cent. pure sialic acid (N-acetyl neuraminic acid) was submitted to the same procedures.

When bovine submaxillary mucoprotein was used as substrate in experiments designed to characterise Cl. welchii neuraminidase, however, Warren's thiobarbituric acid assay gave erratic results. This was subsequently considered to be due to the fact that Warren's method gives no colour with the di- and tri-acetyl neuraminic acid predominant in bovine submaxillary mucin (Blix, 1961 - personal communication).

2. Egg-white mucin. Graphs of the absorption spectra of the Bial orcinol reaction products obtained with the egg-white substrate and acid-hydrolysed egg-white showed ill-defined maxima at 580 mμ (fig. 3). The Svennerholm (1957) modification of the Bial reaction yielded a product with a very poorly defined extinction peak at 580 mμ; both of these tests were relatively insensitive. Graphs of the results obtained following the thiobarbituric acid

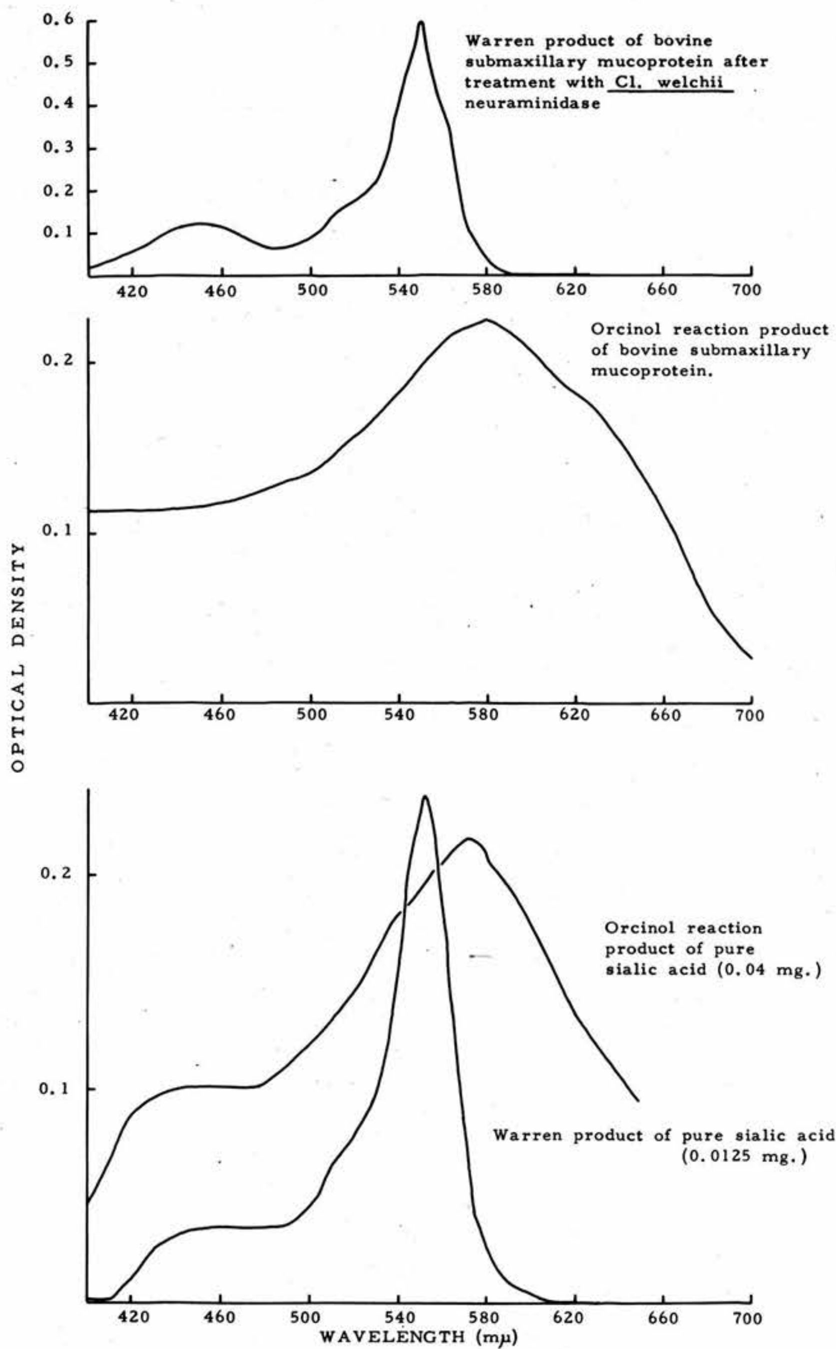


Fig. 2. Absorption spectra of chromogens (orcinol and Warren) in untreated and Cl. welchii-treated bovine submaxillary mucoprotein compared with the spectra obtained with pure N-acetyl neuraminic acid.

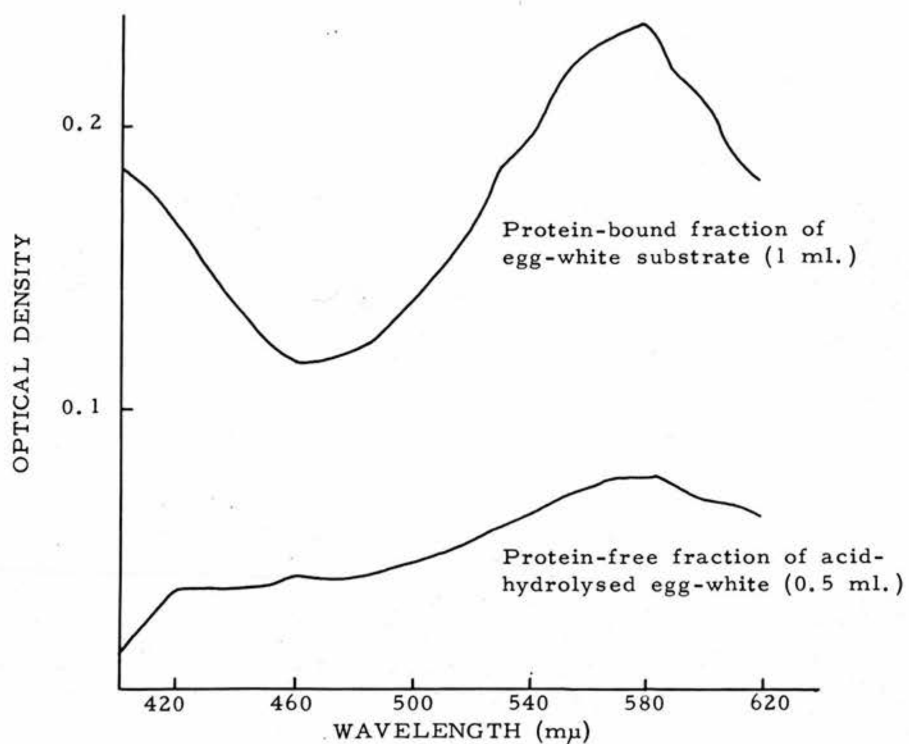


Fig. 3. Absorption spectra of chromogens (orcinol reaction) in the precipitated fraction of untreated egg-white substrate and in the supernatant fraction of acid-hydrolysed egg-white after precipitation of protein in each case with phosphotungstic acid.

reaction with hydrolysed egg-white, Cl. welchii neuraminidase-treated egg-white and pure sialic acid, confirm Warren's claim that his method is much more sensitive and more specific (fig. 4).

Investigation of detectable sialic acid in Cl. welchii and culture media

Samples (1 ml.) of 48-hr blood agar cultures of strains L2A and B, each harvested in 5 ml. saline, were subjected to the orcinol reaction. Graphs of the absorption spectra of the products were compared with a graph of the absorption spectrum of the orcinol reaction product obtained with bovine submaxillary mucoprotein which contains protein-bound sialic acid (Fig. 5). There is no evidence of the presence of protein-bound or free sialic acid in the Cl. welchii culture products.

The Warren thiobarbituric acid reaction revealed no free sialic acid when tests were performed directly on 48-hr nutrient broth culture supernates or disintegrated bacterial deposits of Cl. welchii. The bacteria obtained from four 48-hour blood agar cultures of strain L2A were harvested in 10 ml. saline and centrifuged. The supernate was retained and the bacterial deposit resuspended in 4 ml. of saline. A 2 ml. volume

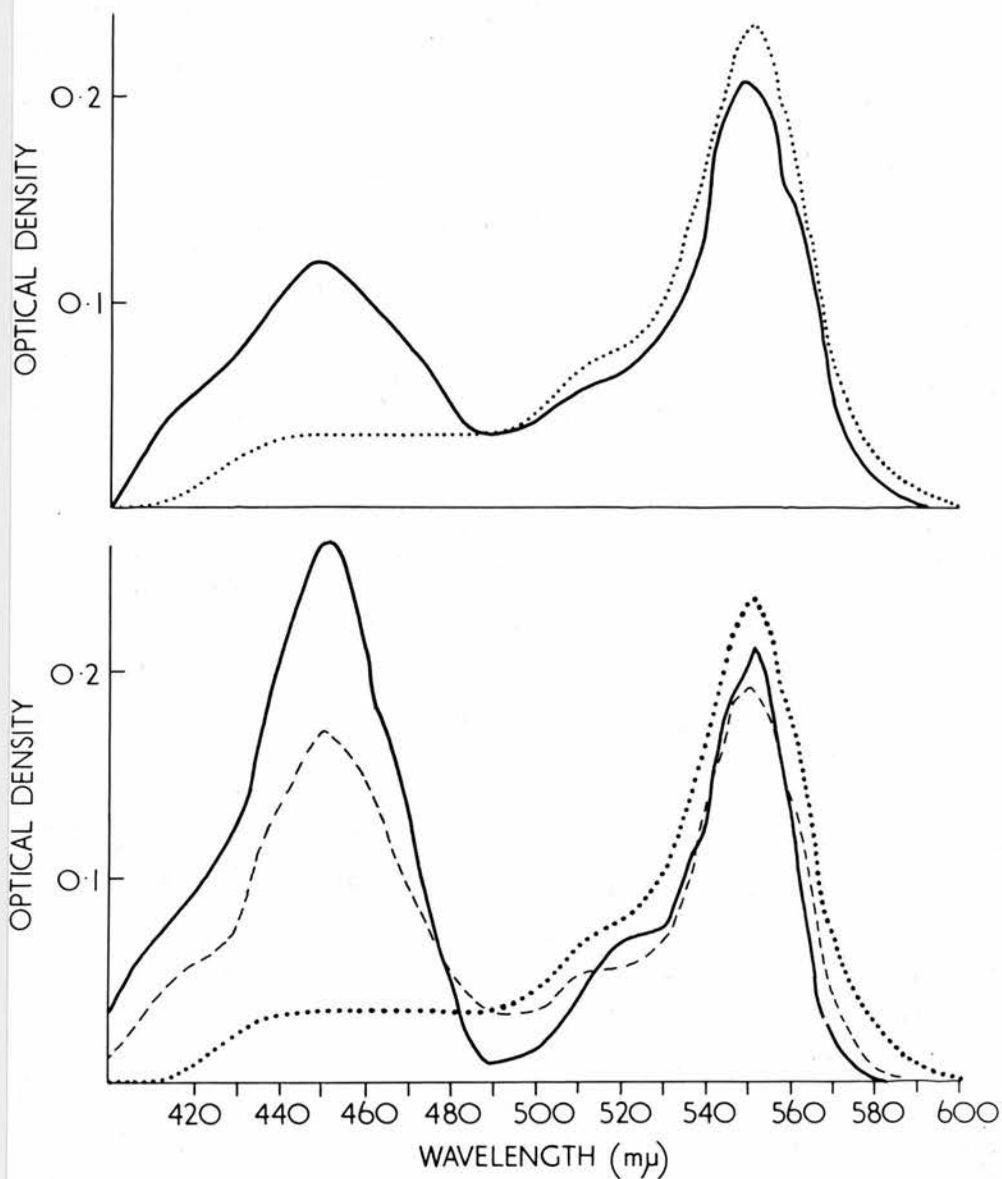


Fig. 4. The upper graph shows absorption spectra of Warren products of acid-hydrolysed egg-white (unbroken line) and pure sialic acid (dotted line). The lower graph shows absorption spectra of Warren products of egg-white substrate treated with <u>Cl. welchii,</u> freshly isolated strain 402 (unbroken line) and stock laboratory strain L2A (broken line), compared with the spectrum obtained with pure sialic acid (dotted line).

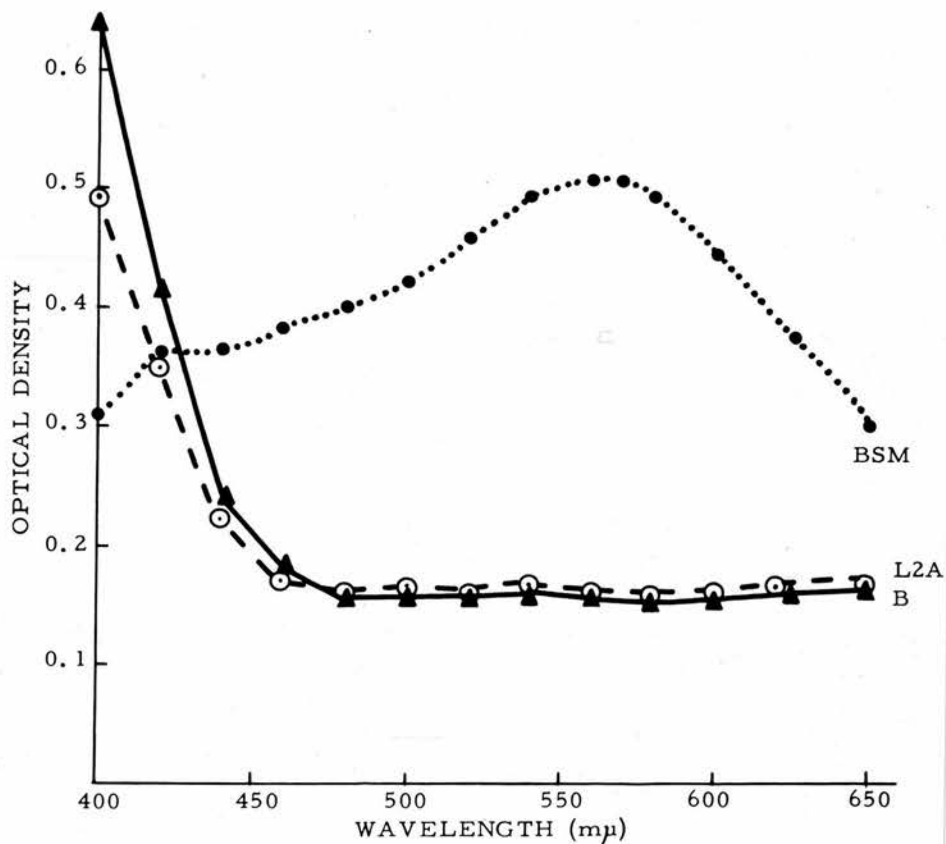


Fig. 5. The absorption spectra of the Bial (orcinol) products obtained with harvested blood agar cultures of Cl. welchii, strains L2A (circles, broken line) and B (triangles, unbroken line) compared with the spectrum of the Bial product of bovine submaxillary mucoprotein (BSM: dotted).

of this dense suspension was treated with the ultrasonic disintegrator. Samples of the supernate, intact bacterial deposit and disintegrated suspension were then hydrolysed by adding equal volumes of 0.2 N sulphuric acid and heating at 80°C. for 1 hr. No sialic acid was detectable in the resulting solutions by the Warren procedure. Treatment of a disintegrated suspension of *Cl. welchii* with *Cl. welchii* neuraminidase did not release detectable sialic acid. These findings do not necessarily exclude the presence of sialic acid in some undetectable form in *Cl. welchii*, but constitute good evidence that the organism does not produce any substance likely to interfere directly in the neuraminidase assay procedure.

Traces of sialic acid were detectable in neat samples of sterile nutrient broth and Ellner medium. Sialic acid residues were certainly detectable in sterile cooked-meat broth, especially after acid hydrolysis. With the possible exception of cooked-meat broth, the detectable sialic acid contributed by the media investigated was minimal and, allowing for the dilution of the culture medium component and the excess of substrate involved in the neuraminidase assay procedure, it would not be detectable under the conditions of the test. 'Time 0' (unincubated)

mixtures of culture product, buffer and substrate were, of course, routinely included in all enzyme assays and it was evident that no significant interference occurred, even when cooked-meat broth cultures were used as the source of neuraminidase.

Enzyme Assay

Release of N-acetyl neuraminic acid by products of Cl. welchii was determined by two methods after incubation with one of the substrates. As the Bial orcinol reaction measures total sialic acid, i.e. bound as well as free sialic acid, the protein-bound component was initially removed from the test sample by precipitation with phosphotungstic acid, as described in the Methods section. The orcinol reaction was used predominantly in association with bovine submaxillary mucoprotein substrate in the earlier stages of this investigation and was useful in confirming results obtained later with the more sensitive Warren assay method. The Warren thiobarbituric acid assay was employed directly to indicate the presence of free sialic acid. The standard assay procedure ultimately adopted and used in most experiments was as follows:

Standard enzyme assay. A mixture of 0.2 ml. of the enzyme preparation to be tested and 0.3 ml. 0.1 M, pH 5.1 Tris-maleate buffer was added to 0.5 ml. egg-white substrate solution. The tube

was incubated for 30 min. or 60 min. as indicated in the text. A 0.2 ml. quantity of the mixture was then assayed directly by the Warren procedure (see Methods) for free neuraminic acid. A sample of the mixture was also assayed at time 0, and a water blank and standard preparation of known free sialic acid content carried through the same procedure simultaneously.

A refinement of this technique recommended by Dr Gunnar Blix (personal communication) and employed by Mayron, Robert, Winzler and Rafelson (1961) was to stop the enzyme reaction and remove protein from the mixture by adding cold phosphotungstic acid. The details of this procedure are noted in the Methods section; it was successfully employed in a detailed investigation of the rate of Cl. welchii neuraminidase activity (q.v.).

Characterisation of Cl. welchii neuraminidase

It was necessary to confirm that the nature of the activity revealed by the enzyme assay which employed egg-white as substrate was consistent with the known facts concerning Cl. welchii neuraminidase activity. The agent releasing neuraminic acid from the egg-white substrate was therefore characterised with regard to the influence of temperature on the rate of reaction,

its activity in the presence of different amounts of substrate, the activity of increasing amounts of enzyme on standard amounts of substrate, the pH optimum and the influence of pH on its thermostability.

Rate of activity. Release of neuraminic acid was marked within a few minutes at 37°C. and was virtually linear with time up to cleavage of more than 80 per cent. of the available substrate (fig. 6). There was reduced but significant activity within 5 - 15 min. at 16°C. Enzyme-substrate mixtures held at 4 - 5°C. showed no significant release of neuraminic acid during 1 - 2 hr but, unless held frozen, transient rises in temperature during handling of such chilled mixtures on the bench allowed some activity to take place.

Effect of increase of substrate. The release of neuraminic acid from increasing amounts of egg-white substrate (0 - 1.5 ml.) by 0.5 ml. aliquots of 48 hr nutrient broth cultures of a laboratory stock strain (L2A) and a fresh isolate (500) was studied. Mixtures of culture and substrate solution were incubated at constant volume in the presence of acetate buffer at pH 5.1 for 1 hr at 37°C.; 0.2 ml. amounts were thereafter assayed by the thiobarbituric acid method for free neuraminic acid. The results (fig. 7)

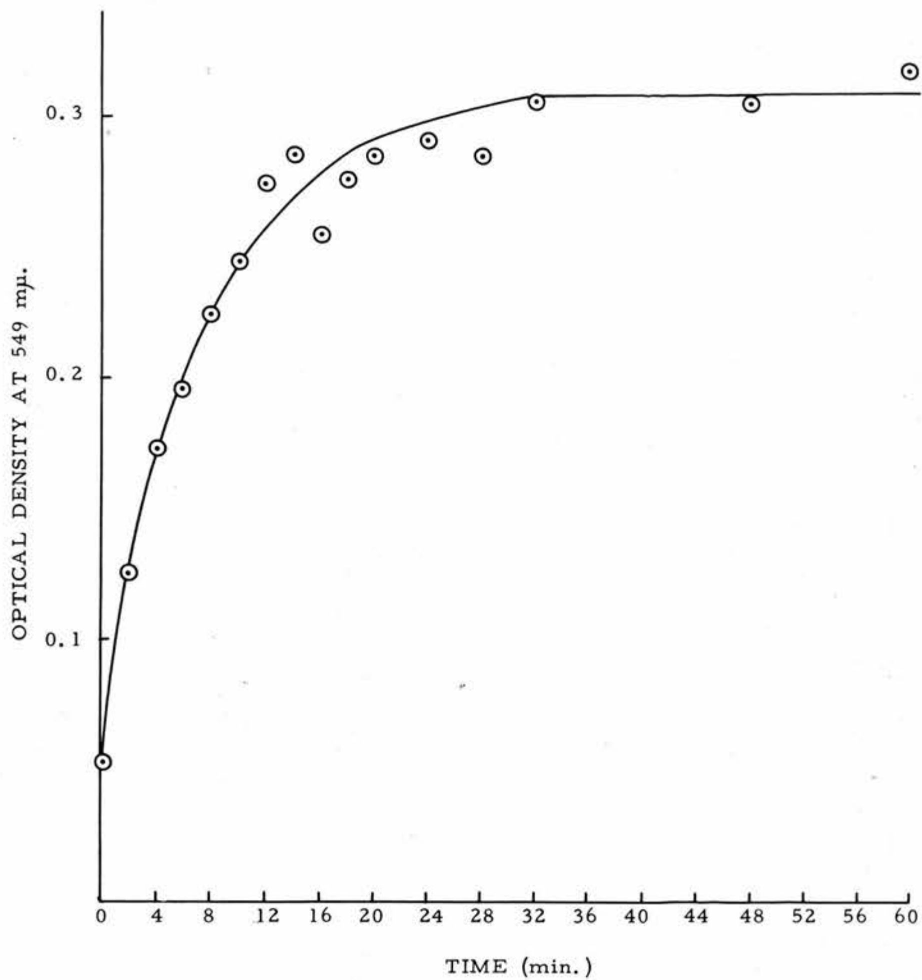


Fig. 6. Release of Warren chromogen from egg-white substrate by dialysed Ellner culture of Cl. welchii, strain L2A, as a function of time; standard assay procedure. Reaction stopped with chilled phosphotungstic acid after various incubation times at 37°C.

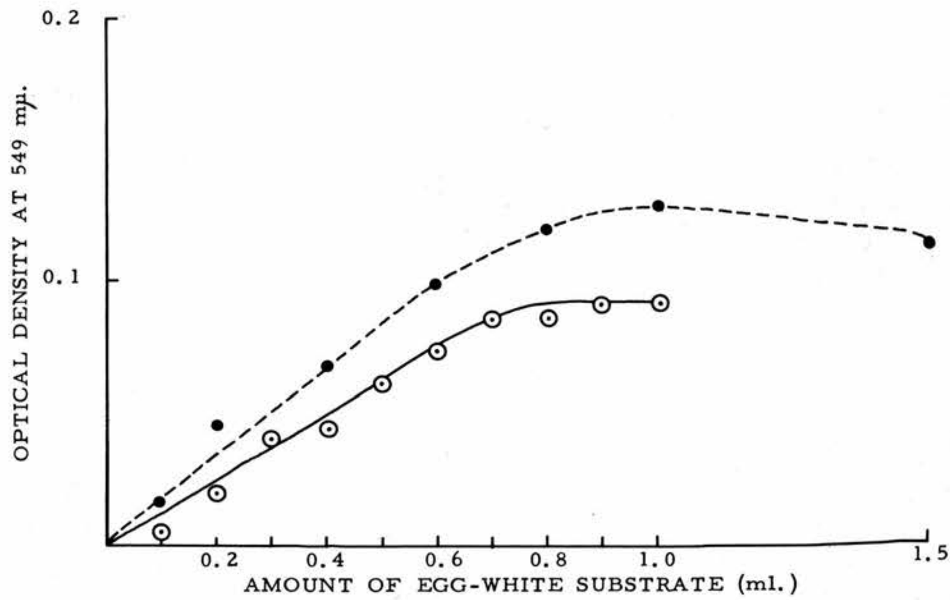


Fig. 7. Release of Warren chromogen by a freshly isolated strain of Cl. welchii (unbroken line) and a laboratory stock strain (broken line) as a function of egg-white substrate concentration.

indicate that enzyme saturation occurred at 0.8 ml. substrate solution in each case.

Effect of increase of enzyme. The release of neuraminic acid from constant amounts (0.5 ml.) of egg-white substrate by increasing amounts of an active Ellner culture supernate of strain L2A on incubation at 37°C., pH 5.1, for 30 min. is illustrated in fig. 8.

Effect of pH on activity. Acid conditions enhanced and alkaline conditions reduced the release of neuraminic acid from egg-white substrate by cultures of *Cl. welchii* at 37°C. Fig. 9 shows that, when tested over a pH range of 5.1 - 8.5, optimal activity occurred at pH 5.1 - 5.6.

Effect of pH on thermostability. The agent releasing neuraminic acid from egg-white substrate was inactivated by heat at 60°C. for 10 min. in all tests over a pH range of 5.1 - 8.5 (Tris-maleate buffers). When exposed to 55°C. for 10 min., inactivation was maximal at alkaline pH values and least at acid pH (5.1) (Table 45). Control tests showed that this was not attributable merely to better activity of residual enzyme at its optimal pH.

There was no evidence of significant lability of the enzyme when held for 2 hr at 37°C. in the presence of Tris-maleate buffers over the pH

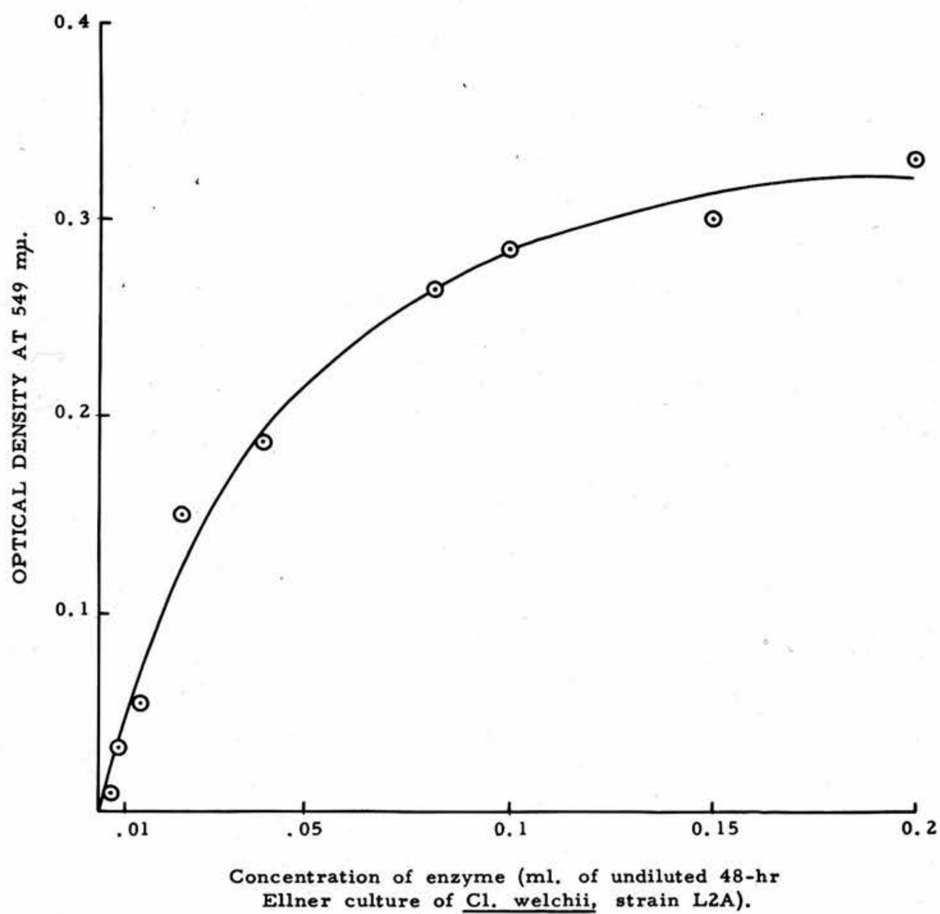


Fig. 8. Release of Warren chromogen from egg-white substrate by Cl. welchii enzyme as a function of enzyme concentration.

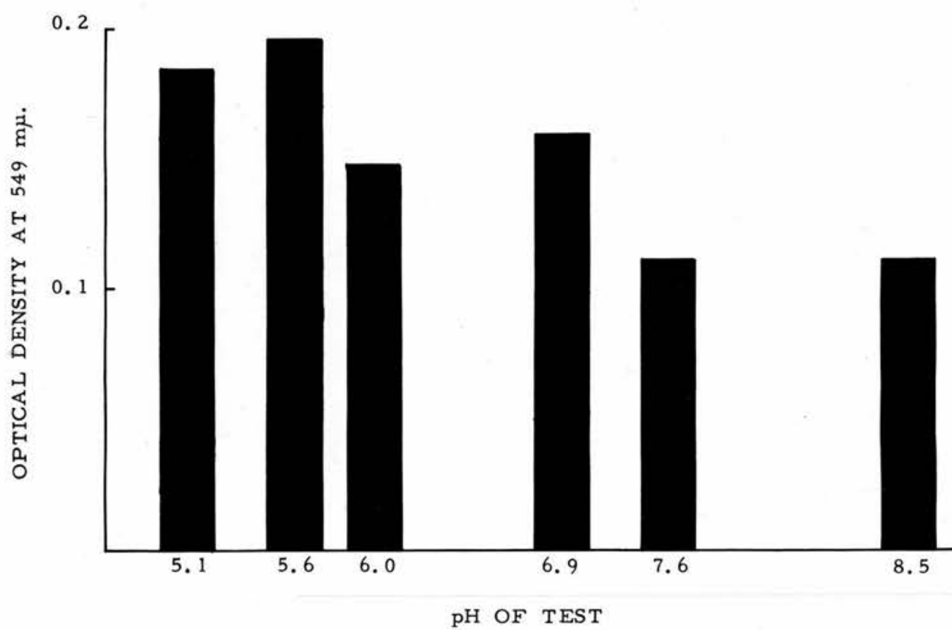


Fig. 9. Effect of pH of test on release of Warren chromogen from 0.4 ml. of egg-white substrate by Cl. welchii enzyme in 0.2 ml. of Ellner culture concentrate. (Reaction at 37°C. for 15 min. in presence of 0.4 ml. of 0.05 M Tris-maleate buffer).

Table 45. Effect of pH on thermolability of *Cl. welchii* enzyme releasing sialic acid from egg-white.

Enzyme preparation heated at 55°C. for 10 min. at pH:	Residual activity (milli-units of neuraminidase per ml.)
5.1	180
6.0	150
6.9	150
7.6	130
7.8	50
8.5	45

range 5.1 to 8.5 in the absence of substrate. The enzyme preserved its activity when stored as frozen culture at -40°C. for at least 14 months; samples held in the refrigerator at 4°C. retained their activity satisfactorily for some weeks.

The activity of the enzyme was not enhanced under reducing conditions. The enzyme was not lost on dialysis in a cellophan bag against distilled water at 4°C. Approximately 30 per cent. of activity was lost on Seitz filtration.

The above results confirmed that the activity demonstrated by the thiobarbituric acid assay of the products obtained from the interaction of _Cl. welchii_ cultures with egg-white substrate was neuraminidase activity. The characteristics of the agent in _Cl. welchii_ cultures which released neuraminic acid from egg-white substrate are those which have been described for _Cl. welchii_ neuraminidase.

Neuraminidase activity. Activity is expressed as units of enzyme, 1 unit being defined as that amount of enzyme which will cleave 1 μ mole of N-acetyl neuraminic acid from an excess of egg-white substrate in 30 min. under the conditions of the standard assay (Molecular weight of N-acetyl neuraminic acid : 309).

The absorptions obtained at wavelengths of 549 and 570 mμ with the products of the Warren and

Bial assays respectively performed on measured amounts of pure N-acetyl neuraminic acid are shown (fig. 10). Colour production was linear, in each case, with concentration of N-acetyl neuraminic acid.

When increasing quantities of an Ellner culture supernate of Cl. welchii strain L2A, rich in neuraminidase, were incubated with 0.5 ml. amounts of egg-white substrate solution in the presence of Tris-maleate buffer at pH 5.1, the content of free N-acetyl neuraminic acid did not exceed 0.013 mgm. in the 0.2 ml. samples subjected to the Warren assay. The amount of available N-acetyl neuraminic acid in 0.5 ml. of the egg-white substrate is thus equivalent to (0.013 x 5) 0.065 mgm. and the standard assay procedure is quantitative for Cl. welchii neuraminidase over the range 0 - 250 milli units per ml. of culture. This degree of sensitivity was considered very suitable for the investigation envisaged in which Cl. welchii cultures were to be screened for neuraminidase activity.

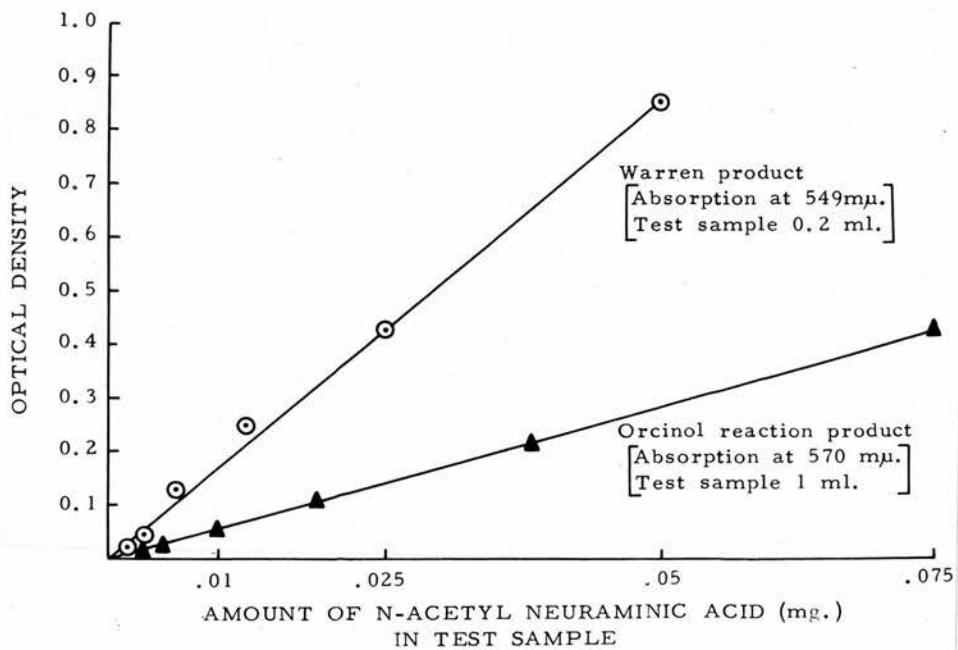


Fig. 10. Absorptions obtained at wavelengths of 549 and 570 mμ with the products of the Warren (thiobarbituric acid) and Bial (orcinol) assays respectively performed on measured amounts of pure N-acetyl neuraminic acid.

The production of neuraminidase by *Cl. welchii* and its relationship to the haemagglutinin and other diffusible products

(i) Influence of cultural conditions. In initial studies with bovine submaxillary mucoprotein as substrate, the relatively insensitive Bial assay revealed no detectable neuraminidase production by old stock strains or freshly isolated strains of *Cl. welchii* when cultured for 20 - 48 hr on blood agar and harvested with saline as for routine haemagglutination tests. Cultures grown for 48 hr in glucose broth were similarly devoid of detectable neuraminidase, as were cultures in the fluid medium recommended by Popenoe and Drew (1957). The Bial assay method in conjunction with the submaxillary mucoprotein substrate was sufficiently sensitive to detect significant neuraminidase activity in a commercial preparation of *V. cholerae* filtrate and also in fourfold concentrates of harvested 48 hr blood agar cultures and Ellner cultures of old stock and freshly isolated strains of *Cl. welchii*.

Results of subsequent work with the more sensitive procedure ultimately adopted as the standard neuraminidase assay method showed that neuraminidase production was best in 48 hr cultures on horse blood agar or in cooked-meat broth. Cultures grown on cellophan-covered

blood agar plates were also rich in neuraminidase and could be harvested free from non-dialysable substances present in the medium. Good yields of neuraminidase were obtained from cultures in nutrient broth or Ellner medium. The neuraminidase content of a 48 hr culture in nutrient broth containing 10 per cent. of serum was low, lower in horse digest broth and minimal in 1 per cent. glucose broth. An enhanced yield of neuraminidase was obtained from a 20 hr culture grown in nutrient broth to which 12 per cent. (v/v) sterile egg-white had been added, but the neuraminidase content was reduced after further incubation for 24 hr to less than that present in the simple 48 hr nutrient broth control culture. Cultures in cooked-meat broth and nutrient broth, the medium generally used for neuraminidase production, contained slightly more neuraminidase at 48 hr than at 24 hr.

Neuraminidase production varied among strains, but freshly isolated strains and old stock strains of *Cl. welchii* were included in those found to produce large amounts of neuraminidase. For example, the neuraminidase activity of an 18 hr nutrient broth culture of stock strain L2A was less than that of freshly isolated strain 402 but greater than that of freshly isolated strain

401. In another experiment, the neuraminidase activity of 20 hr nutrient broth cultures of five freshly isolated strains of Cl. welchii varied from the relatively low figure of 80 milli-units per ml. to the relatively high figure of 200 milli-units per ml. None of the freshly isolated strains produced haemagglutinin.

All of the old stock strains which produced haemagglutinin also produced neuraminidase. Although freshly isolated strains produced neuraminidase in the absence of haemagglutinin, no strain was ever encountered which produced haemagglutinin in the absence of detectable neuraminidase. There was, however, no apparent quantitative correlation between production of haemagglutinin and neuraminidase. For example, Table 46 shows the results of an experiment in which the neuraminidase and haemagglutinating activities of washings obtained from 48 hr blood agar cultures of several stock strains of Cl. welchii were compared. Strain F 6599/59 produced much neuraminidase and much haemagglutinin; strain L7C produced a similar amount of neuraminidase but much more haemagglutinin; strain L9D produced a similar amount of neuraminidase but much less haemagglutinin; strain L8D produced much less neuraminidase but more

Table 46. A comparison of the haemagglutinating and neuraminidase activities of 48-hr cultures of ten strains of *Cl. welchii*.

Strain	Reciprocal of haemagglutinin titre	Neuraminidase activity (milli-units per ml.)
L7C	256	150
F 6599/59	32	150
L8D	16	70
B	8	140
L1A	8	140
L9D	4	140
F 4637/60	4	140
A_3	NIL	120
F	NIL	NIL
300	NIL	185

haemagglutinin; and strain A on this occasion produced no haemagglutinin but yielded a significant amount of neuraminidase.

(ii) Diffusibility of neuraminidase. Neuraminidase activity of broth cultures of *Cl. welchii* was associated with the centrifuged bacteria-free supernatant fluids and not with their washed bacterial deposits. Although disintegration of the bacterial deposit of a haemagglutinating culture released much haemagglutinin, disintegration of washed bacterial deposit from a non-haemagglutinating neuraminidase-rich culture did not release neuraminidase; but some neuraminidase activity was associated with those disintegrated bacterial deposits which were powerfully haemagglutinating. For example, a 48 hr nutrient broth culture of strain L2A yielded a washed bacterial deposit which, after disintegration and resuspension, had a haemagglutinin titre of 1 in 64 and a neuraminidase content of 30 milliunits per ml. Concentrated thrice-washed bacterial deposit obtained from three 48 hr blood agar cultures of the same strain had, after disintegration, a haemagglutinin titre of 1 in 2048 and a neuraminidase content of 70 milliunits per ml.

Seitz filtration of a haemagglutinating

neuraminidase-positive culture supernatant reduced its neuraminidase activity by only 30 per cent., although this completely removed its haemagglutinating activity.

These findings indicate that the haemagglutinin is not identical with the neuraminidase of <u>Cl. welchii</u>, but there is evidence of a 'one-way' correlation in the occurrence of these two factors.

(iii) <u>Fractionation of Cl. welchii cultures</u>

(a) <u>Precipitation with ammonium sulphate</u>. In a preliminary experiment, 300 ml. of centrifuged supernate from pooled 18 hr Ellner cultures of stock strain L2A were concentrated tenfold on courulose at 4°C., dialysed thereafter in cellophan against tapwater at 4°C. for 24 hr and against distilled water at 4°C. for a further 24 hr, and then precipitated in fractions at 18°C. (initial pH: 7.3) by stepwise addition of solid ammonium sulphate. The successive precipitates were recovered by centrifugation and resuspension in 10 ml. amounts of distilled water. These fractions were then dialysed overnight against a large excess of distilled water at 4°C. to remove the ammonium sulphate before investigating their haemagglutinin, lecithinase and neuraminidase content. The results (Table 47) showed that

Table 47. The results of ammonium sulphate fractionation of an 18-hr Ellner culture concentrate of *Cl. welchii*, strain L2A, showing the relative content, in each fraction, of lecithinase haemagglutinin and neuraminidase.

Fraction No.	Percentage saturation range of $(NH_4)_2SO_4$ precipitating fraction	Relative content, in each fraction, of		
		Lecithinase	Haemagglutinin	Neuraminidase
1	0 - 40	-	+	-
2	40 - 50	+++	+++	±
3	50 - 60	++	++	++
4	60 - 70	-	-	+++
5	70 - 80	-	-	±
6	80 - 100	-	-	-

+++, ++, + = decreasing amounts.
± = doubtful positive. - = nil detectable.

this was a useful method for the initial separation and concentration of these factors.

Subsequent experiments involved fractionation of 500-1000 ml. amounts of centrifuged supernates obtained from nutrient broth cultures of a freshly isolated strain (300) and a laboratory stock strain (L2A). Solid ammonium sulphate was added in amounts calculated to give a series of precipitates at 0 - 40, 40 - 50, 60 - 70 and 70 - 80 per cent. ammonium sulphate saturation (Dixon, 1953). These precipitates were separately collected by centrifugation (6,500 g for 30 min. at 4°C.) at each stage. The precipitates were resuspended in 20 - 25 ml. amounts of distilled water and dialysed in cellophan sacs for 1 - 2 days against a large excess of distilled water at 4°C. The distilled water was changed 2 - 3 times and dialysis was enhanced by agitation produced by an aerating pump. The protein content of the fractions was then assessed spectrophotometrically and the haemagglutinating, lecithinase and neuraminidase activities of each were determined.

Ammonium sulphate treatment of 1000 ml. of the centrifuged supernate from a 20 hr nutrient broth culture of strain 300, freshly isolated from faeces, yielded virtually no precipitate at 40 per cent. ammonium sulphate saturation within

30 min. at 18°C. Five fractions were subsequently obtained and investigated in detail. These included the original supernate '0' and the precipitates obtained at 0 - 50, 50 - 60, 60 - 70 and 70 - 80 per cent. ammonium sulphate saturation, labelled fractions 1, 2, 3 and 4 respectively (Table 48). Lecithinase activity was most concentrated in fraction 1 but demonstrable in fractions 1, 2 and 3. Neuraminidase activity was most concentrated in fractions 2 and 3. Haemagglutinating activity was absent, with the exception of a doubtful positive trace in fraction 2. Toxicity tests in mice showed that fraction 1 was lethal on intravenous injection of doses down to 0.05 ml., whereas fraction 3 which was rich in neuraminidase produced no effect in doses up to 0.4 ml.

Fractions obtained after ammonium sulphate precipitation of 500 ml. of a supernate obtained by centrifugation of pooled 3-day nutrient broth cultures of stock strain L2A were similarly investigated but each precipitation step was allowed to take place in the cold for several hours; the procedure occupied 3 days. In this case the original culture supernate showed slight haemagglutinating activity, slight lecithinase activity and moderate neuraminidase activity.

Table 48. The relative content of lecithinase, haemagglutinin and neuraminidase in four fractions obtained from a 20-hr nutrient broth culture of a freshly isolated strain of *Cl. welchii*.

Fraction No.	Percentage saturation range of $(NH_4)_2SO_4$ precipitating fraction	Relative content, in each fraction, of		
		Lecithinase	Haemagglutinin	Neuraminidase
1	0 - 50	++++	-	+
2	50 - 60	+++	±	+++
3	60 - 70	+	-	+++
4	70 - 80	±	-	+

++++, +++, + indicate decreasing amounts

± = doubtful positive. - = nil detected.

Precipitates were obtained at ammonium sulphate saturation levels of 0 - 40, 40 - 50, 50 - 60, 60 - 70, 70 - 80 and 80 - 100 per cent., and the resultant fractions were labelled 1 - 6 respectively. Table 49 shows that fraction 1 had no haemagglutinin, much lecithinase and a little neuraminidase. Fraction 2 had much haemagglutinin, a moderate amount of lecithinase and a relatively large amount of neuraminidase. Fraction 3 had much haemagglutinin, slight lecithinase activity and good neuraminidase activity. Fraction 4 had virtually no haemagglutinin or lecithinase but significant neuraminidase activity.

These results show that neuraminidase can exist quite independently of detectable haemagglutinin, and that neuraminidase activity does not necessarily parallel lecithinase activity. Lecithinase can occur in the presence of neuraminidase without producing haemagglutinating activity; but haemagglutinin has never been demonstrated in the absence of neuraminidase activity from the original whole culture.

(b) Chromatography. Initial attempts to fractionate *Cl. welchii* culture supernates with calcium phosphate and with carboxymethyl cellulose in columns were unsuccessful, but the use

Table 49. The relative content of lecithinase, haemagglutinin and neuraminidase in six fractions obtained from a 3-day nutrient broth culture of a stock laboratory strain of *Cl. welchii*.

Fraction No.	Percentage saturation range of $(NH_4)_2 SO_4$ precipitating fraction	Relative content, in each fraction, of		
		Lecithinase	Haemagglutinin	Neuraminidase
1	0 - 40	++++	-	+
2	40 - 50	+++	++++	+++
3	50 - 60	+	+++⊥	+++⊥
4	60 - 70	-	-	++⊥
5	70 - 80	-	-	⊥
6	80 - 100	-	-	-

++++, +++⊥, +++, ++⊥, ++, +⊥, + indicate decreasing amounts
⊥ = doubtful positive. - = nil detected.

of columns containing diethylaminoethyl (DEAE) cellulose produced promising results.

A column, 7.5 cm. x 1 cm., of DEAE-cellulose was equilibrated with 0.02 M phosphate buffer at pH 7.2 and then loaded with 10 ml. of a concentrated 48 hr Ellner culture supernate of strain L2A which had been dialysed against the same buffer for 48 hr at 4°C. The column was thereafter eluted with the phosphate buffer and an increasing molarity gradient of sodium chloride from 0 - 2 M (see Methods). Haemagglutinin was eluted over the molarity range 0.8 - 1.0 (Table 50) coincident with release of protein from the column. The fractions which possessed haemagglutinating activity showed receptor-inactivating and neuraminidase activity in proportion to their haemagglutinin content. A-enzyme activity was also associated with the haemagglutinating fractions.

When the active fractions were pooled, reconcentrated and eluted again from another DEAE-cellulose column under the same conditions as before, haemagglutinin, neuraminidase and receptor-inactivating agent were again released virtually simultaneously.

Attempts to obtain more effective separation of these substances by using longer columns were

Table 50. A comparison of the activities present in seven fractions eluted from a DEAE-cellulose column loaded with material obtained from a 48-hr Ellner culture of Cl. welchii, strain L2A.

Fraction number	Reciprocal of haem-agglutinin titre	Reciprocal of titre of receptor-inactivating agent	Relative neura-minidase content	Relative A-enzyme content
10	nil	nil	±	-
12	nil	4	+	+
14	nil	64	+++	/
16	128	2048	++++	+++
18	32	256	++++	+++
20	4	32	+++	+
24	nil	nil	-	/

+, +++, ++++ = increasing degrees of activity.

± = doubtful positive.

- = no activity demonstrable.

/ = test not done.

at first frustrated by release of non-specific haemagglutinating material from the cellulose. This complication was avoided by pre-eluting the column with strong sodium chloride solution and by prolonged washing of the column with phosphate buffer.

Fig. 11 illustrates the results of an experiment in which 15 ml. of a concentrated supernate obtained from a 48 hr Ellner culture of strain L2A was eluted from a 10 cm. DEAE-cellulose column under the conditions described above and the fractions were investigated for protein content, lecithinase activity, neuraminidase and haemagglutinin. Lecithinase was released before the main yields of haemagglutinin and neuraminidase which were again in close association, but a peak of neuraminidase activity was detected in fractions relatively devoid of haemagglutinin.

Two peaks of neuraminidase activity were also encountered in fractions obtained on a subsequent experiment when 8 ml. of tenfold concentrated supernatant of a 72 hr nutrient broth culture of strain L2A was eluted from a 14.5 cm. column of DEAE-cellulose. As on the previous occasion, the intervening fraction showing low neuraminidase activity contained the maximum amount of protein.

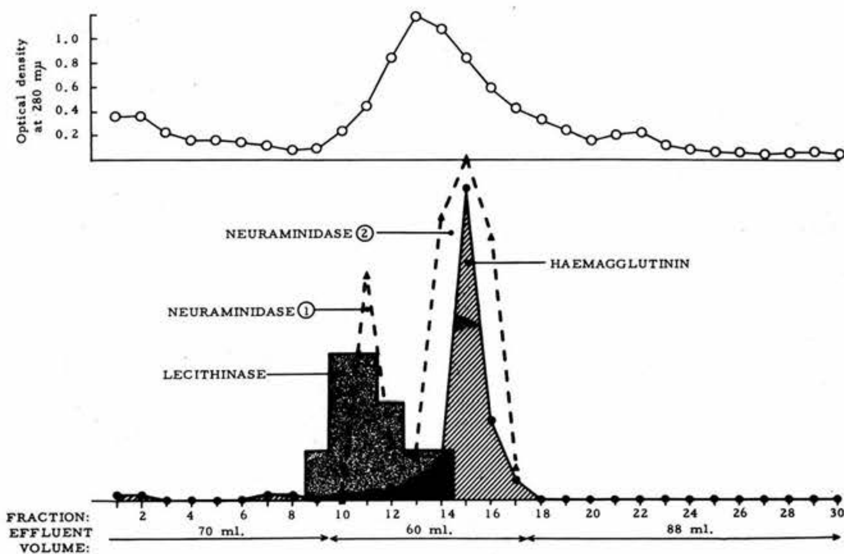


Fig. 11. A semi-diagrammatic representation of the relative protein, lecithinase, neuraminidase and haemagglutinin content of fractions eluted from a DEAE-cellulose column loaded with material from a 48-hr Ellner culture of Cl. welchii, strain L2A.

(c) Combined fractionation. The above results indicated that precipitation was extremely fine at 40 - 50 per cent. ammonium sulphate saturation. It was thus found possible to fractionate 1000 ml. of crude 48 hr nutrient broth culture supernate of strain L2A into five fractions, of which the third fraction (precipitated overnight at 4°C. at 50 - 60 per cent. ammonium sulphate saturation and centrifuged in the cold thereafter at 2000 g for 30 min.) contained most of the haemagglutinin, neuraminidase and lecithinase (Table 51). This fraction was dialysed exhaustively against 0.02 M phosphate buffer, pH 7.2, at 4°C. It was then centrifuged free of deposit and 8 ml. loaded on to a DEAE-cellulose column, 12 cm. x 1 cm., which had been eluted for 24 hr with the same phosphate buffer. Elution with phosphate buffer thereafter with an increasing sodium chloride molarity gradient yielded fractions in which there was partial separation of A-enzyme from haemagglutinin, and haemagglutinin from lecithinase. Haemagglutinin and neuraminidase were again eluted in fairly close association*. Receptor-inactivating agent was released in association with neuraminidase but was detectable in subsequent fractions showing no apparent neuraminidase activity (q.v.).

* see Fig. 12.

<u>Table 51</u>. <u>The results of ammonium sulphate fractionation of a 48-hr nutrient broth culture supernate of *Cl. welchii*, strain L2A, showing the relative content, in each fraction, of lecithinase, haemagglutinin and neuraminidase.</u>

Material used	Percentage saturation range of $(NH_4)_2SO_4$ precipitating fraction	Relative content, in each fraction, of:		
		Lecithinase	Haemagglutinin (reciprocal of titre)	Neuraminidase (milli-units per ml.)
Original culture		+	8	100
Fraction				
1	0 - 40	+	4	70
2	40 - 50	++	16	135
3	50 - 60	++++	128	215
4	60 - 70	±	4	175
5	70 - 80	-	NIL	20

++++, ++, + = decreasing amounts
± = doubtful positive
\- = no lecithinase detectable

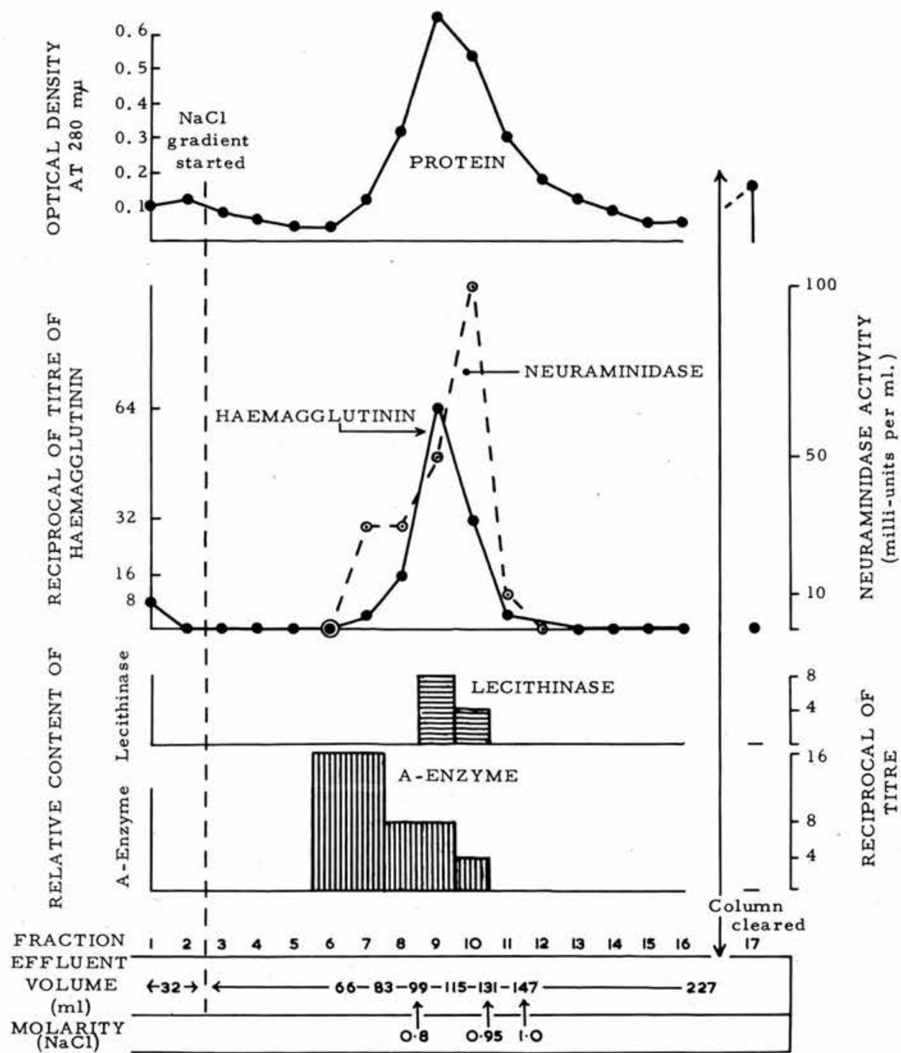


Fig. 12. Composite diagram showing results of column chromatography of fraction 3 (Table 51). The haemagglutinin, neuraminidase and lecithinase components are closely associated.

These results confirm that the enzyme destroying the A substance of peptone (A-enzyme) is not identical with the haemagglutinin or the neuraminidase of Cl. welchii. Haemagglutinating activity has again been demonstrated in the absence of lecithinase, but it is evident that lecithinase, neuraminidase and haemagglutinin appear to occur in close association in Cl. welchii cultures. Neuraminidase and haemagglutinin are particularly closely related and it seems that the receptor inactivating agent is also closely related to neuraminidase.

Studies on the interaction of the haemagglutinin, neuraminidase, receptor-inactivating agent and lecithinase of Cl. welchii.

(i) Absorption by human red cells. The absorption of the haemagglutinin, neuraminidase, receptor-inactivating agent and lecithinase from a concentrated culture supernate by human group-A red cells was studied under different conditions of temperature and pH. Aliquots (4 ml.) of tenfold concentrated 3-day nutrient broth culture of strain L2A were each mixed with the centrifuged red cell deposits obtained from 10 ml. amounts of a 1 per cent. red cell suspension in saline in the presence of 0.5 ml. of 0.05 M Tris-maleate buffer at the pH and temperature indicated and held for 5 min. Thereafter, the mixtures were centrifuged and the activities of the supernates compared with the activities of the original unabsorbed concentrate. The red cell deposits were, in turn, resuspended in 3.5 ml. amounts of saline buffered with 0.5 ml. of Tris-maleate at pH 8.5, held for 15 min. at 16°C., and then centrifuged to obtain the eluates. As it was desired to avoid any possible inhibitors of activity at the red cell surface, calcium sequestering agents were not added during these procedures. It is difficult to obtain clean

results in experiments of this kind involving haemolytic systems, but the results (Table 52) show quite clearly that haemagglutinin was absorbed under all conditions tested, optimally at acid pH in the cold, whereas lecithinase, neuraminidase and receptor-inactivating agent were apparently not so readily absorbed. Haemagglutinin was not subsequently eluted from the red cells but small amounts of neuraminidase, receptor-inactivating agent and lecithinase occurred in the eluates.

When a larger amount of red cells was used, absorption of the receptor-inactivating agent was easily demonstrable. Absorption of 4 ml. of a centrifuged Ellner culture supernate of strain L2A with 1 ml. packed washed human group-A red cells at 4°C. removed the receptor-inactivating agent almost completely. The mixture was held for only 1 min., but the centrifugation procedure occupied a further 10 - 15 min. before the absorbed supernate was removed for testing. Absorption occurred equally well at pH 5.1 and 8.5. Absorption of neuraminidase under these conditions was less complete. The samples from which receptor-inactivating agent was removed retained considerable neuraminidase activity.

These findings show that haemagglutinin has

Table 52. The influence of pH and temperature on the absorption of various diffusible products of *Cl. welchii* by human group-A red cells under certain conditions (see text).

Substance tested	Reciprocal of haem-agglutinin titre	Neuraminidase activity (milli-units per ml.)	Reciprocal of lecithinase titre	Reciprocal of receptor-inactivating agent titre
Original unabsorbed	16	120	16	64
after absorption: at pH 5.1, 4°C.	nil	100	16	64
at pH 5.1, 16°C.	nil	45	16	64
at pH 8.5, 16°C.	4 - 8	50	8	64
in unbuffered saline at 16°C.	4	70	16	64

a greater affinity for the red cell surface than the other factors investigated. Although the receptor-inactivating agent behaves in many ways like the neuraminidase, it does not appear to be identical with active neuraminidase.

(ii) The influence of haemagglutinin on lecithinase activity. Human red cells (blood group-A) were strongly agglutinated with *Cl. welchii* haemagglutinin and then exposed to *Cl. welchii* lecithinase to determine if the haemagglutinin, which is generally produced by relatively avirulent stock strains, may protect cells against lecithinase activity. A 3 ml. amount of a 2 per cent. suspension of red cells was mixed with 3 ml. of a disintegrated bacterial suspension of strain L2A containing 1024 haemagglutinating units per ml. at 17°C. and held for 30 min. A control sample of the red cell suspension was similarly treated with saline in place of haemagglutinin. The red cells in each case were then resuspended by shaking and 0.5 ml. aliquots added in parallel to two series of doubling dilutions of a haemagglutinin-negative lecithinase-rich fraction obtained from a culture of *Cl. welchii* strain L2A by ammonium sulphate fractionation. The diluent was calcium chloride 0.001 per cent. in physiological saline. The tests were shaken,

incubated for 1 hr at 37°C. and held overnight at 4°C. The results (Table 53) showed definite haemolysis of the haemagglutinin-treated red cells in dilutions of the lecithinase fraction up to 1 in 16, but the control red cells showed definite lysis in dilutions of the lecithinase fraction up to 1 in 64. As these findings may be explained on the basis of protection of red cells against access of haemolysin by prior agglutination, the following experiment was performed.

A 2 ml. amount of the lecithinase fraction was mixed with 2 ml. of haemagglutinin and held at 17°C. for 30 min. A control mixture contained the lecithinase fraction and saline. Both were then serially diluted each in two series of parallel doubling dilutions in saline containing calcium chloride 0.001 per cent. To one of each series was added 0.5 ml. aliquots of a solution containing lecitho-vitellin (LV), and to the other, 0.5 ml. aliquots of human group-A red cells in a 1 per cent. suspension in physiological saline. All of the tests were shaken, incubated at 37°C. for 1 hr and thereafter held at 4°C. overnight. The results of these tests (Table 54) show no inhibition of the lecithinase by the haemagglutinin. Indeed, there is a

Table 53. The effect or prior admixture with haemagglutinin on the haemolytic activity of lecithinase against human red cells.

Reagent mixture	Haemolysis produced by reagent mixture diluted 1 in:						
	4	8	16	32	64	128	256
Lecithinase plus saline	C	C	AC	AC	D	D	-
Lecithinase plus haemagglutinin	C	C	C	C	D	D	-

C = complete lysis; AC = almost complete lysis
D = definite lysis; - = no haemolysis detectable

Table 54. The effect of prior admixture with haemagglutinin on lecithinase activity with egg-yolk suspension as substrate.

Reagent mixture	Opalescence produced by reagent mixture diluted 1 in:						
	4	8	16	32	64	128	256
Lecithinase plus saline	+++	++	±	-	-	-	-
Lecithinase plus haemagglutinin	+++	++±	+	±	-	-	-

+++, ++±, ++, + = decreasing degrees of opalescence.
± = doubtful positive.
- = no opalescence produced.

slight potentiation of the lecithinase in both of the tests to which haemagglutinin was added.

(iii) The influence of the haemagglutinin on neuraminidase activity. This cannot be ascertained with certainty as haemagglutinin is associated so closely with neuraminidase activity. When 0.3 ml. of a powerfully haemagglutinating disintegrated suspension of strain L2A was incubated for 30 min. with 0.2 ml. of a neuraminidase-rich fraction mixed with 0.2 ml. egg-white substrate and 0.3 ml. Tris-maleate buffer at pH 5.1, no inhibition of neuraminidase activity occurred. Control tests showed that the haemagglutinating suspension itself possessed considerable neuraminidase activity and the result is therefore of little significance.

(iv) The relationship of the lecithinase to the receptor-inactivating agent. The lecithinase fraction used in (ii) above was found to produce no receptor-inactivating effect. When human group-A red cells were exposed to doubling dilutions of this fraction for 2 hr at 18°C. they remained fully agglutinable by Newcastle disease virus.

(v) Inhibition of haemagglutinin and neuraminidase by antiserum. Inhibition of 8 - 10 haemagglutinating units of Cl. welchii haemagglutinin

in 0.5 ml. amounts of saline was produced by 0.5 ml. aliquots of commercial _Cl. welchii_ type-A antiserum in initial dilutions of antiserum up to 1 in 128. Non-specific inhibition was demonstrable with normal rabbit serum in dilutions up to 1 in 32 and commercial _Cl. oedematiens_ type-A antiserum in dilutions up to 1 in 8. The commercial _Cl. welchii_ antiserum also inhibited the neuraminidase. Control tests using normal rabbit serum and _Cl. oedematiens_ antiserum showed no inhibition; on the contrary, tests incorporating these sera showed exceptionally high readings for liberated sialic acid. These findings illustrate a further complication in the serological approach to this study and suggest that protein-bound sialic acid in animal sera is a more accessible substrate for _Cl. welchii_ neuraminidase than the bound sialic acid occurring in egg-white.

The Transforming Activity of Cl. welchii cultures

A detailed investigation of the factor in cultures of Cl. welchii which induces panagglutinability of red cells was not envisaged in the present study. As there is much confusion in the literature concerning the nature of the factor responsible for the Thomsen-Friedenreich panagglutination phenomenon, and as no particular technique or standard organism has been universally accepted for its demonstration, it is probable that further confusion will arise until the mechanism of the Thomsen-Friedenreich reaction is fully investigated. Current interest in mechanisms of panagglutination and their possible participation in clinical syndromes justifies a brief report of some of the significant characteristics of the panagglutination reactions observed during the present work. The terms 'transformation' and 'transforming principle' now have several different connotations in microbiology but are used here to denote the alteration of the red cell and the factor producing that alteration associated with the Thomsen-Friedenreich panagglutination phenomenon.

Diffusibility of transforming principle.

The transforming principle of *Cl. welchii*, like the haemagglutinin, is essentially diffusible. Residual activity was almost completely removed from washed centrifuged bacterial deposits by repeated washing with physiological saline; this produced a parallel decrease in haemagglutinating and transforming activity. Both activities were thereafter restored and greatly enhanced when the repeatedly washed bacterial deposit was disintegrated ultrasonically.

Demonstration of panagglutinability.

Transformation was demonstrated by mixing aliquots of a 1 per cent. suspension of human group-A red cells in physiological saline and the *Cl. welchii* culture material, holding at 18 - 20°C. for 2 hr and thereafter adding 2 drops of the mixture to 1 drop of blood group-B antiserum or *Cl. welchii* antiserum on a porcelain tile which was gently rocked. Agglutination of the red cells caused by any *Cl. welchii* haemagglutinin present was usually minimal and is not clearly demonstrable by the tile test although, in the absence of a control test, it may be mistaken for a weak panagglutination reaction by an inexperienced observer. Clumping of red cells associated with panagglutination is coarse and easily

recognised.

Human group-A red cells were rendered apparently panagglutinable after exposure to certain Cl. welchii cultures for about 15 min. prior to adding blood group-B antiserum. This 'rapid transformation' occurred at 4°C. as well as at 18°C. and more consistently at these lower temperatures than at 37°C. If the red cells, culture material and serum were all added simultaneously, the development of this panagglutination reaction was delayed and reduced in degree. These findings indicated that rapid transformation may be associated with an adsorption mechanism.

Zone effects were frequently encountered in tests in which the first two or three of a series of doubling dilutions of a powerfully transforming culture showed no demonstrable modification of the red cells although strong induction of panagglutinability was demonstrable at higher dilutions. This suggested that there may be competition for or inhibition of transforming factors or receptor sites at the lower dilutions of crude culture material.

Rapid transforming activity was particularly associated with haemagglutinating cultures. The dilution at which transformation was demonstrable

was always higher than that at which haemagglutinin could be detected; thus, sub-haemagglutinating doses of a haemagglutinating culture conferred panagglutinability. Transforming activity was less readily demonstrated with non-haemagglutinating cultures of Cl. welchii. Red cells mixed with centrifuged saline-harvested blood agar culture supernates of freshly isolated (non-haemagglutinating) strains of Cl. welchii showed no development of panagglutinability when the mixtures were made and held at 4°C.; there was slow development of panagglutinability (within 5 hr) in mixtures held at 20 - 22°C., and more rapid development of panagglutinability (within 30 min.) at 37°C. When 0.5 ml. of packed washed human group-A red cells was used to absorb 2.5 ml. of a fourfold concentrated and dialysed Ellner culture supernate of a non-haemagglutinating strain, the red cells were rendered panagglutinable. The procedure occupied 20 min. at 20°C. and, as considerable haemolysis had occurred, a further 30 min. at 20°C. elapsed while the red cells were washed twice with physiological saline prior to testing for panagglutinability. When this experiment was repeated at 4°C. throughout, the red cells were not rendered immediately panagglutinable;

these red cells became panagglutinable after standing overnight at 18°C.

Influence of pH.

Rapid transforming activity of Cl. welchii saline-harvested blood agar culture was easily demonstrable over the pH range 6.0 - 7.5. It was slightly reduced at pH 5.6 and definitely diminished at pH 8.2. Optimal activity occurred within the pH range 6.8 - 7.5, i.e. the red cells were sensitised within 15 min. of adding Cl. welchii culture and were thereafter strongly agglutinated within 10 - 15 min. of adding antiserum.

Inactivation and inhibition studies.

The rapid transforming activity of Cl.welchii was thermolabile at 60°C. within 10 min. at acid pH values but there was some slight protective effect against heat-inactivation under alkaline conditions. The presence of sterile supernate from cooked-meat broth (containing inhibitor C) certainly slowed the development of the rapid panagglutination reaction but did not inhibit it completely. The addition of 1 per cent. peptone solution (inhibitor A) rendered the agglutination less coarse but did not inhibit the reaction.

Fractionation studies.

In the combined fractionation studies of *Cl. welchii* culture products reported earlier, the elution of the transforming principle from a DEAE-cellulose column was also investigated by testing the red cells for panagglutinability after overnight exposure to serial dilutions of the various fractions at 18 - 20°C. The results (Table 55) show that transforming activity occurred in fractions removed from the column exactly in parallel with the receptor-inactivating agent. The pattern of elution of neuraminidase was very similar although weak transformation and receptor inactivation were produced by fractions devoid of detectable neuraminidase activity. Transforming activity did not exactly parallel haemagglutinating activity as, for example, fraction 9 contained more haemagglutinin but less transforming principle than fraction 10. A-enzyme activity was not correlated with transforming activity.

FRACTION	HAEMAGGLUTINATION produced by dilutions of 1 in:								RECEPTOR-INACTIVATION produced by dilutions of 1 in:								PANAGGLUTINABILITY induced by dilutions of 1 in:								Relative A-ENZYME content
	4	8	16	32	64	128	256	512	4	8	16	32	64	128	256	512	4	8	16	32	64	128	256	512	
5	⊥	−	−	−	−	−	−	−	+++	+++	+++	+++	+++	+++	+++	+++	−	−	−	−	−	−	−	−	/
6	⊥	−	−	−	−	−	−	−	+++	+++	+++	+++	+++	+++	+++	+++	−	−	−	−	−	−	−	−	+++
7	+++	−	−	−	−	−	−	−	(+++)	+++	+++	+++	+++	+++	+++	+++	+	−	−	−	−	−	−	−	+++
8	ℓ	+++	+++	⊥	−	−	−	−	ℓ	⊥	(+++)	+++	+++	+++	+++	+++	ℓ	+	+	−	−	−	−	−	++
9	L	L	L	+++	+++	−	−	−	L	L	L	⊥	(+++)	+++	+++	+++	L	L	L	+++	+++	+++	−	−	++
10	L	ℓ	+++	+++	−	−	−	−	L	ℓ	(+++)	(+++)	−	−	−	⊥	L	ℓ	+++	+++	+++	+++	+++	++	+
11	++	+	−	−	−	−	−	−	(+++)	(++)	+	−	+++	+++	+++	+++	++	++	+++	+++	+	−	−	−	−
12	−	−	−	−	−	−	−	−	+++	⊥	+++	+++	+++	+++	+++	+++	+++	++	+	−	−	−	−	−	−
13	−	−	−	−	−	−	−	−	−	⊥	+++	+++	+++	+++	+++	+++	+++	++	⊥	−	−	−	−	−	−
14	−	−	−	−	−	−	−	−	⊥	+++	+++	+++	+++	+++	+++	+++	+++	−	−	−	−	−	−	−	−

+, ++, +++ = increasing degrees of agglutination of red cells
⊥ = doubtful positive
− = no agglutination
L = test result obscured by complete haemolysis
ℓ = partial haemolysis

"Negative" readings in this column indicate significant myxovirus (NDV) receptor-inactivation.
Brackets surround results attributable to residual effect of Cl. welchii haemagglutinin

Positive readings here denote red cells rendered panagglutinable.

+, ++, +++ = increasing amounts of A-enzyme activity.
/ = test not done

Table 55. A comparison of the content of haemagglutinin, receptor-inactivating agent, transforming principle and A-enzyme in various fractions of a Cl. welchii culture eluted from a DEAE- cellulose column.

It seems likely that two different mechanisms of transformation participate in these systems. One appears to involve sensitisation of the red cell by an adsorption process and this induces panagglutinability within about 15 min. at room temperature or in the cold. It is associated with the presence of the *Cl. welchii* haemagglutinin and it may be that red cells brought together by haemagglutinin have their normal mutually repellent character sufficiently altered to render their surfaces susceptible to non-specific agglutinins or conglutinins in sera. Blockage of acidic sialic acid radicals at the red cell surface by adsorption of haemagglutinin or neuraminidase is likely to alter the reactivity of the red cell surface. As the haemagglutinin has been shown to be closely associated with residual neuraminidase activity, prolonged exposure under suitable conditions may unmask T-antigens so that red cells rapidly altered by the adsorption process could later become truly transformed by enzymatic activity. Results of preliminary studies suggest, however, that enzymatically induced transformation of the red cell surface is blocked in the presence of a high concentration of haemagglutinin. For example, an Ellner culture supernate of strain L2A

possessing strong haemagglutinating and neuraminidase activity produced rapid transformation of red cells at 4°C. and 18°C., but only weak transformation of red cells at 37°C. The red cells did not become more strongly panagglutinable on exposure to this culture supernate for 1 hr at 37°C. Red cells similarly exposed at 37°C. to non-haemagglutinating, weakly neuraminidase-positive cultures, on the other hand, gradually became strongly panagglutinable within 30 min. Blockage of neuraminidase by high concentrations of haemagglutinin may explain the zone phenomena encountered in serial dilution tests of transforming activity of *Cl. welchii* cultures.

These findings indicate that rapid transformation, an adsorption phenomenon associated with the *Cl. welchii* haemagglutinin, is not demonstrable at room temperature with freshly isolated (non-haemagglutinating) strains although these strains produce neuraminidase which is almost certainly responsible for the less rapidly apparent Thomsen-Friedenreich phenomenon.

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SECTION IV : Discussion

DISCUSSION

'The experimental method must not let too many machines get between it and the whole, and must find some way of putting the fragments surgically removed for experimental purposes back into the whole. The comparative method is frequently in position to restrain the generalizations deduced from the experimental procedures, and to keep the experimenter from steering away from the goal which is an understanding of the totality.'

Theobald Smith (1934).

Although studies of red cells in test tubes may yield valuable information, it must be accepted that tests of red cell reactions in physiological saline may be very unphysiological. In addition, some tests demonstrating certain phenomena with red cells as substrate exploit mechanisms which *in vivo* are unlikely to take place at or in red cells; they may, for example, be more likely to involve epithelial cells. Investigations into the action of purified microbial products on purified substrate material may yield much more apparently valid information, but in this case it can be argued that many biological processes are dynamically inter-related and do not occur naturally in isolation. It is

therefore necessary to be cautious in assessing the significance of the results of such studies and the warning of Theobald Smith was aptly reiterated in this context by M.G. Macfarlane (1955).

The primary aim of the present work has been to resolve some of the confusion regarding the identity of certain diffusible products of Clostridium welchii associated with the haemagglutinin, to examine their interaction at the red cell surface and to elucidate some of the phenomena recorded by previous workers in this and related fields. In the course of the investigation many other phenomena of interest have been observed. It has seemed necessary to attempt to find explanations for several of those which were considered of particular practical importance for the further development of this work. The findings will be discussed initially with regard to their significance in defining the factors of Cl. welchii causing haemagglutination, destruction of blood group-A substance, myxovirus receptor destruction and induction of red cell panagglutinability. The experimental evidence is now adequate to justify modification of existing hypotheses concerning these phenomena. There is much less adequate experimental data

available with which to develop theories regarding the interaction of these factors at cell surfaces or to evaluate their importance in possible mechanisms of pathogenicity _in vivo_. For example, although it is reasonable to presume that neuraminidase activity may be of survival value to an organism involved in processes of mammalian tissue decomposition, we remain uncertain if this product is elaborated by the organism in its pathogenic rôle and in its natural environment. There is, however, a fair amount of circumstantial evidence upon which to base some guarded speculation concerning mechanisms of cell surface attack by _Cl. welchii_ and this will be submitted in the latter part of the discussion when the further development of this work is considered.

While the processes involved in the production of gas-gangrene are of great interest, it is important to bear in mind that our ignorance of the host-parasite relationship with reference to _Cl. welchii_ is reflected in many facets. It is evident in our lack of understanding of the apparent immunity of intestinal epithelium to attack by commensal _Cl. welchii_, in our lack of knowledge regarding the host growth control exerted by _Cl. welchii_ when this organism is introduced into the gut of germ-free animals (see

Lev, 1961), in our uncertainty regarding enterotoxaemic conditions caused by Cl. welchii in livestock and in our ignorance of the actual mechanism of Cl. welchii food-poisoning in man. It is known that proteolytic enzymic activity occurring in the intestine can inactivate Cl. welchii lecithinase (Goudie, 1959). Lecithinase interferes with energy-producing oxidizing systems present in the mitochondria of cells (M.G. Macfarlane and Datta, 1954) and may exert a toxic depressive effect on growth following absorption from the gut of young animals. Destruction of our alimentary epithelium may be countered by rapid epithelial regeneration and minimised by the presence of protective mucus and the occurrence of regular peristalsis. The protection afforded by antitoxins and toxoids against the Cl. welchii enterotoxaemias in animals indicates that direct toxic effects underlie these conditions. It may be that the simple mechanics of unusually active growth of a large inoculum of Cl. welchii organisms, protected from the gastric acid barrier by the protein in which they are ingested, produce the symptoms associated with Cl. welchii food-poisoning. These are all reasonable comments relevant to the problems outlined above. Nevertheless, the

host-parasite relationship begins and is often determined at the cell surface; hypotheses regarding cell-surface attack by Cl. welchii may be of value in stimulating further research in this important field.

The haemagglutinin of Cl. welchii has been characterised as a factor possessing affinity for a wide range of red cells and active over a wide pH range (5.5 - 9.4) and temperature range (4° - 37°C.). It is partly diffusible. Free haemagglutinin is probably liberated as a result of autolysis since it appears in ageing cultures and much of the haemagglutinin remains associated with the intact washed bacterial cell. The haemagglutinin is unlike an enzyme in being active at 4°C. and in the presence of 0.5 per cent. formaldehyde. Moreover, it is not readily eluted from its substrate at 37°C. If it is an enzyme, as Wickham suggests, its haemagglutinating activity does not seem to depend on the completion of its enzymatic activity. The results of the adhesion experiments confirm that the bacilli are not involved as links between the red cells in haemagglutination by bacillary deposit. The bacilli probably cause haemagglutination by further liberation of diffusible haemagglutinin. It seems that susceptible red cells rapidly

adsorb multivalent haemagglutinin particles, even in the cold, and these provide rather delicate molecular linkages between them. These linkages tend to be disrupted when the temperature is raised to 37°C., but there is no evidence that this is due to enzymatic decomposition of the haemagglutinin receptor at the red cell surface. It may be that the molecular configuration of the haemagglutinin or its receptor substance is reversibly altered on heating to 37°C. or that thermal agitation at this temperature interferes with haemagglutination.

The theory postulated by Wickham that the haemagglutinin and the enzyme destroying blood group-A substance (A-enzyme) are the same, was partly based upon her assumption that their mutual inhibition by cooked-meat broth is due to a single inhibitor, namely blood group-A substance (inhibitor A). Among the different strains of _Cl. welchii_ investigated in the present study, ability to produce haemagglutinin and ability to destroy inhibitor A were apparently related, but the activities of the two factors were susceptible to different inhibitors, the C and A inhibitors derived from cooked meat and peptone respectively. Semi-purified blood group-A substance, a powerful inhibitor of blood group-A isoagglutination, did

not inhibit the haemagglutinin. Moreover, cultural conditions that suppressed haemagglutinin production did not suppress A-enzyme production. The haemagglutinin is distinguishable from A-enzyme in being active with red cells of all ABO groups, in being much more stable than the A-enzyme described by Schiff (1939), in having pH optima different from that of the A-enzyme characterised by Stack and Morgan (1949), and in being eluted from DEAE-cellulose in a slightly different manner. For example, one fraction was obtained which showed strong A-enzyme activity in the absence of haemagglutinating activity whereas another fraction rich in haemagglutinin possessed only very weak A-enzyme activity.

It is also clear that the haemagglutinin and the neuraminidase (RDE) of Cl. welchii are not identical. The haemagglutinin differs from the Cl. welchii RDE described by McCrea (1947) in having an optimum pH of 6.8, in being less thermostable in acid than in alkaline conditions and in being unable to be eluted spontaneously from red cells at 37°C. If the haemagglutinin and RDE were identical, as Wickham suggested, the two factors would share a common substrate at the red cell surface. McCrea considered the RDE of Vibrio cholerae virtually identical with the

Cl. welchii RDE and therefore active against the same substrate. My finding that red cells stripped of myxovirus receptors by *V. cholerae* RDE are highly sensitive to *Cl. welchii* haemagglutinin indicates that the substrate of influenza virus and *V. cholerae* RDE at the red cell surface is not identical with that of the *Cl. welchii* haemagglutinin. If *V. cholerae* RDE and *Cl. welchii* RDE are identical, then this suggests that *Cl. welchii* RDE and *Cl. welchii* haemagglutinin are not identical, but the parallelism noted in the present work between inhibition of the myxovirus and *Cl. welchii* haemagglutinins implies that their substrates are closely related. This, in turn, suggests that the substrates of the haemagglutinin and the RDE of *Cl. welchii* are either identical or closely related.

The neuraminidase assay procedure developed in the present investigation confirmed that *Cl. welchii* neuraminidase can exist in the absence of haemagglutinating activity. Non-haemagglutinating freshly isolated strains consistently produced neuraminidase. These strains also produced lecithinase so that, despite Burnet's reported observation (McCrea, 1947) that lecithinase causes agglutination of red cells treated

with _V. cholerae_ filtrate, the co-existence of lecithinase and neuraminidase in _Cl. welchii_ cultures does not account for the _Cl. welchii_ haemagglutinin. The fractionation studies have also demonstrated that the neuraminidase and the haemagglutinin are not identical, but the two factors are eluted simultaneously when they occur together. Although neuraminidase can exist in the absence of haemagglutinin, haemagglutinin has never under any circumstances been demonstrated in a culture which lacks neuraminidase. Mucoprotein inhibitors of the haemagglutinin contain the substrate of neuraminidase, and treatment of mucoprotein inhibitors with _V. cholerae_ filtrate containing neuraminidase inactivates the inhibitor of the _Cl. welchii_ haemagglutinin. Antisera which inhibit the haemagglutinin specifically also inhibit the neuraminidase. My finding that the haemagglutinin was not produced by any of more than 100 freshly isolated strains of _Cl. welchii_ confirms the experiences of Katitch (1954) and Wickham (1956a) and suggests that, in its haemagglutinating form, it is not a naturally occurring product of the organism. It may be significant that the maximum yields of haemagglutinin occurred in ageing and sporing cultures, and it is probable that the haemagglutinin may represent

a degraded form of one or more enzymes normally active on cell-surface polysaccharides. The available evidence indicates that the haemagglutinin may be an altered form of neuraminidase which has lost its ability to decompose its substrate and become spontaneously eluted from the red cell surface although it still retains affinity for the receptor substance.

There is no doubt that the haemagglutinin is much more readily adsorbed than neuraminidase to susceptible red cells and, if one can assume that it is neuraminidase which is responsible for enzymatic induction of panagglutinability, there is some evidence that haemagglutinin may block access for enzymatically active neuraminidase at the receptor sites. The haemagglutinin is also adsorbed to a greater extent than neuraminidase on to Seitz filter pads. A reasonable hypothesis based on the experimental findings is that haemagglutinin may exist as non-homogeneous aggregates or particles of neuraminidase which have multivalent affinity for substrate but, because of some variation in molecular configuration or as a result of the size of the complex, cannot complete the enzymatic decomposition of the receptor material to which they become adsorbed.

The finding of a receptor-inactivating agent

in <u>Cl. welchii</u> cultures is of interest, especially as this factor is not temperature dependent. The results of the fractionation studies indicate that this is most probably a form of neuraminidase and that it can block myxovirus receptor sites in the cold without necessarily destroying the receptor substance enzymatically. It may be that receptor-inactivation is merely a more sensitive index of the presence of neuraminidase than the enzyme assay, but there are some discrepancies in the present work if this theory is correct. It seems quite possible that altered neuraminidase may exist in relatively small inactive complexes of a size which would not allow of haemagglutinating activity. This would explain the occurrence of minimal receptor-inactivating activity in fractions devoid of demonstrable neuraminidase and haemagglutinating activity.

Mayron, Robert, Winzler and Rafelson (1961) have recently demonstrated that it is possible to obtain neuraminidase free of haemagglutinating activity from influenza virus preparations. These authors suggest that the neuraminidase and haemagglutinin sites on the virus may be different but closely related entities. If this can be confirmed it is evident that some of the concepts

of myxovirus-host cell interaction will require slight modification, but the findings with *Cl. welchii* in the present study suggest that it may be worth while to consider the alternative that haemagglutinin is an altered form of neuraminidase.

There are several objections to Wickham's view that *Cl. welchii* cultures induce panagglutinability of red cells by destruction of specific blood group substance at their surfaces and that the enzyme destroying blood group-A substance is the *Cl. welchii* RDE. Wickham's theory would require that all three enzymes of *Cl. welchii* active against blood group substances A, B and O (H), possessed receptor-destroying and panagglutinating activity. McCrea characterised a single enzyme as the *Cl. welchii* RDE, and this did not appear to induce panagglutinability. Moreover, Stack and Morgan (1949) found no enzyme able to inactivate blood group substances in culture filtrates of *V. cholerae*. In addition, red cells rendered panagglutinable, presumably by RDE activity (Burnet, McCrea and Stone, 1946) but not necessarily so (Stewart, Steele and Martin, 1959), do not lose their group-specific antigens (Bird, 1954); and the non-group-specific T receptor involved in panagglutination is not related to

the ABO antigens (Sanger and Race, 1958).
Burnet, McCrea and Stone noted that panagglutinability is produced by Friedenreich's strain M (diphtheroid) without loss of virus receptors. Indeed, it seems unlikely that the Cl. welchii A-enzyme could induce panagglutinability in the manner suggested by Wickham, because this enzyme was found by Morgan (1947) to be incapable of destroying the natural blood group-A substance of intact red cells although it is active on the isolated substance and on its relatively denatured polysaccharide residue (inhibitor A) in peptone. Blood group-A substance actually appears to be locked into the lipid or ganglioside of the red cell and is there neither soluble nor accessible to attack by bacterial enzymes which can decompose the isolated substance. Thus, there is a considerable amount of evidence that the A-enzyme of Cl. welchii is not the transforming principle, the neuraminidase or the haemagglutinin of the organism.

It will be evident, nevertheless, that some of the facts quoted to support this argument do not appear to be consistent with my earlier assumptions (i) that the neuraminidases of Cl. welchii and V. cholerae are virtually identical and (ii) that enzymatic transformation of red

cells, i.e. induction of panagglutinability, is almost certainly caused by neuraminidase. The results of the fractionation studies have resolved any doubt that the A-enzyme, the haemagglutinin and the neuraminidase of Cl. welchii are different, but it is advisable to comment at this point that more information is required before the demonstration of inactivation of blood group-A activity of peptone by the A-enzyme of Cl.welchii is finally accepted as proof of the presence of the enzyme which destroys blood group-A substance. The results of my preliminary studies on the Thomsen-Friedenreich phenomenon now indicate a possible reason for the conflicting reports in the literature regarding the induction of panagglutinability by different organisms; the adsorption and enzymatic processes involved in the two mechanisms of induction of panagglutinability discovered in Cl. welchii cultures merit further investigation so that unnecessary confusion in this field is avoided.

The rôle of neuraminidase in the economy of Cl. welchii is not known. The enzyme can be inhibited by suramin (Collee, unpublished result) and this substance has been shown to inhibit a lysozyme-like enzyme involved in bacterial cell separation processes (Lominski, Cameron and

Wylie, 1958) resulting in the production of long chains by various organisms including Cl. welchii when they are grown in the presence of suramin (Lominski and Gray, 1960). In the present investigation, however, it has not been possible to demonstrate the presence of sialic acid residues in Cl. welchii and, although this may be because sialic acid is incorporated in a form undetectable by the methods used, it is doubtful if neuraminidase plays any part in cell separation processes. No neuraminidase production was demonstrated by any of the typical food-poisoning strains of Cl. welchii examined. These strains grow as rapidly as classical strains of Cl. welchii type-A in vitro (Collee, Knowlden and Hobbs, 1961) and do not typically occur in chain formation. The facts therefore indicate that the production of extracellular neuraminidase is not essential for the normal metabolism of the organism in laboratory media.

In the early stages of this work it was thought that the Cl. welchii haemagglutinin might be associated with a bacterial adhesive mechanism and that the haemagglutinin may be involved in Cl. welchii food-poisoning in which an affinity of these strains for epithelial surfaces was considered as a possible preliminary to cell

surface attack by neuraminidase. The results of the experiments show that neither haemagglutinating nor neuraminidase activities are characteristic of typical food-poisoning strains and that the haemagglutinin does not confer adhesive properties on the bacteria.

Gottschalk (1960) thought that the wide distribution of neuraminidase among micro-organisms inhabiting the respiratory and intestinal tracts suggests that the enzyme has been evolved as part of a mechanism which ensures that the organism is not separated from its environment by mucin and is therefore a survival enhancement factor. Consideration of the wide occurrence of sialic acids in animal mucoproteins and ground substance prompted Dewitt and Rowe (1961) to postulate that one or more of the bacterial enzymes concerned in the metabolism of sialic acid complexes might be capable of action against mammalian substrates and thus be associated with mechanisms of virulence.

If some of the mechanisms of pathogenicity of <u>Cl. welchii</u> are to be ascribed to its neuraminidase, it is tempting to speculate how this enzyme, which appears to be non-toxic on intravenous injection in mice, may contribute to the initiation and symptomatology of infections due

to *Cl. welchii*, type A, in man. M.G. Macfarlane (1955) considered that the effects of injection of *Cl. welchii* toxin into animals, e.g. oedema, haemorrhage, local necrosis and shock, as well as the clinical signs of gas-gangrene, can reasonably be attributed to the effect of lecithinase on cell membrane permeability. She pointed out, however, that in general it has not been demonstrated that these effects are initiated or accompanied by decomposition of lecithin (Oakley, 1943, 1954; Zamecnik, Nathanson and Aub, 1947). Oakley, Warrack and Clarke (1947) thought that something besides the enzymic attack on lecithin was involved in the haemolysis of different types of red cells.

The mode of action of *Cl. welchii* lecithinase in producing toxaemia *in vivo* is still in doubt, although much is known about this toxin to support the many hypotheses which have been suggested. Evans (1943a, b; 1945; 1947) showed that, in infections due to *Cl. welchii* type A, the lecithinase is the most important lethal factor and anti-lecithinase (alpha antitoxin) is of prime importance in protection against the lethal effect of *Cl. welchii*. He found that there was a general correlation between production of lecithinase *in vitro* by

strains of Cl. welchii type A, and their ability to kill guinea-pigs, but some feeble lecithinase producers were virulent and some active lecithinase producers were avirulent. This state of affairs has been reviewed and discussed extensively by van Heyningen (1955) who considered that the significant poison formed in a Cl. welchii infected muscle may not be lecithinase; the 'muscle poison' may be an unrecognised product of the organism or something formed by the action of the organism or its products on constituents of muscle. van Heyningen recognised five possibilities, of which the three suggestions relevant to the present discussion are:
(i) The hypothetical muscle poison is produced independently of the lecithinase. (ii) The action of the lecithinase results in the production of the muscle poison. (iii) The toxaemia is due both to the lecithinase and the muscle poison.

McCrea (1947) found that there was a highly significant correlation, in the range of Cl. welchii toxin preparations examined by him, between their lecithinase and cell-modifying activity. The cell-modifying factor had the characteristics of the Cl. welchii neuraminidase, and the results of the present work confirm that

McCrea correlated the lecithinase and neuraminidase activities of his preparations. The cell-modifying activity was inhibited by high dilutions of several *Cl. welchii* antisera in approximate proportion to their anti-lecithinase titre. McCrea subsequently demonstrated that cell-modifying activity was only slightly inhibited by a purified anti-lecithinase antiserum and he postulated that the cell-modifying factor (neuraminidase) and lecithinase are normally produced in parallel by *Cl. welchii* so that antibodies to both enzymes tend to occur in similar proportions in most *Cl. welchii* antisera.

M.G. Macfarlane (1955) has pointed out that comparative studies of the lecithinases produced by different species of clostridia indicate that toxicity is not inherent in lecithinases of a particular biochemical type. She held that an important factor related to toxicity of lecithinase may be the resultant of the rate of action of the enzyme and the rate of repair of the cell surface and that this is dependent primarily on the relationship between the enzyme and the substrate *in situ* in the cell. The *Cl. welchii* lecithinase is more active against red cells of sheep and man than red cells of the horse. The haemagglutinin shows a similar pattern of

affinity and it appears to be closely related to the neuraminidase. It therefore seems relevant to ask if the closeness of fit (and therefore perhaps the degree of toxicity) of the *Cl. welchii* lecithinase is partly dependent on the presence of neuraminidase. If my observation that the haemagglutinin seems to block the access of neuraminidase at the red cell surface is valid, the degree of toxicity of the lecithinase may be considered to be not only dependent on the presence of neuraminidase but also upon the absence of haemagglutinin. Haemagglutinating strains of *Cl. welchii* occur after prolonged laboratory subculture and are then much reduced in their virulence. At present it seems reasonable to believe that, by attacking surface components of cells, neuraminidase may render them more susceptible to lecithinase; but much work remains to be done before it can be assumed that van Heyningen's third preposition holds the truth.

In some cases of classical type A *Cl. welchii* infection in man, notably in septicaemic cases following septic abortion, intravascular haemolysis may occur before toxaemia is manifest (Hill, 1950), whereas intravascular haemolysis was not a feature of cases of severe gas-gangrene investigated by MacLennan and

R.G. Macfarlane (1945). van Heyningen (1955) pointed out that the absence of intravascular haemolysis in such cases indicates that there could hardly be a lethal quantity of toxin in circulation, because (and here he presumably argued by analogy with animal experiments) the amount of toxin necessary to produce haemolysis on intravenous injection is less than the lethal dose. However, as it was inconceivable that _Cl. welchii_ growing profusely in damaged muscle should not produce a considerable amount of toxin, he considered how the evidence of several workers in this field might account for the apparent loss of detectable toxin in wound exudates or extracts of infected tissue. van Heyningen was unable to resolve the conflicting data but he significantly stressed that the only hope of saving life in cases of gas-gangrene lies in surgical removal of the affected tissue. He concluded that a source of poison was removed at operation and that the poison did not seem to be the lecithinase.

A reasonable hypothesis which might resolve many apparently contradictory reports is that neuraminidase production by _Cl. welchii_ _in vivo_ could alter tissue so that lecithinase is more rapidly fixed than _in vitro_ experiments suggest.

This view is consistent with the finding of R.G. Macfarlane and MacLennan (1945) that toxin was adsorbed on living tissues but was found to be readily recoverable after injection into dead tissue; neuraminidase activity in the warm muscle of a living animal is likely to be much greater than in the dead animal. A further consequence of neuraminidase production in the living host may be the exposure of deep antigens, a process analogous to the Thomsen-Friedenreich reaction which has been demonstrated in the present work. There is already experimental evidence, reviewed in the introduction to this thesis (pp. 59 - 60), to suggest that sensitisation induced by absorption of such altered tissue products or transformed red cells may precipitate a dangerous reaction *in vivo*. For example, T antibodies may react with transformed red cells to agglutinate them and subsequently lyse them in the presence of complement (Stone, 1947); transfusion of transformed red cells produces a shock syndrome in the experimental animal (Neter, 1956). It is also possible that elution of neuraminidase from red cells in the circulation would release the enzyme to attack capillary endothelium and intercellular material at sites distant from the focus of infection and thus render these sites particu-

larly susceptible to circulating lecithinase. This type of mechanism may well play a part at some stage of a severe Cl. welchii infection.

Results obtained by Bullen and Cushnie (1962) indicate that in experimental gas-gangrene an inverse relationship may exist between the invasiveness of a strain of Cl. welchii and the protection afforded against it by antiserum. Antiserum gave good protection in a host where invasiveness was poor; in a host where invasiveness was pronounced, antiserum did not give good protection unless circumstances simultaneously favoured limitation of growth of the organism. The factor or factors determining invasiveness are unknown and are likely to be affected in part by local conditions in vivo which influence the growth of the organism. Bullen et al. (1961) considered, for example, that an oxygenated environment or a fairly high eH was essential for the growth-inhibiting effect of antiserum on Cl. welchii growing in embryonated eggs. Antiserum did not produce bacteriostasis when Cl. welchii was growing under more markedly reducing conditions.

In discussing the relationship of the diffusible products of the organism to its invasiveness, Bullen et al. (1962) remarked upon the large number of highly virulent strains which entirely lack

ability to produce diffusible factors like collagenase and hyaluronidase. It is evident that other workers are currently attempting to find a more satisfactory explanation for the mechanism by which _Cl. welchii_ initiates infection in man. Fredette, Forget, and Vinet (1962) attributed the aggressiveness of _Cl. welchii_ cultures to the presence of a hitherto unheeded antigen, unhappily termed "bursting factor" (Fredette and Frappier, 1946), which produces gross swelling when injected intramuscularly into the thigh of a guinea-pig. It is non-toxic but confers aggressive properties on washed _Cl. welchii_ bacilli. Although the factor is said to have been characterised and to differ from collagenase, further characterisation is obviously required. Fredette _et al._ (1962) did not examine their cultures for neuraminidase, but the properties of neuraminidase and "bursting factor" appear to be different. Whether neuraminidase plays any part in conferring invasive properties on _Cl. welchii_ is still unknown. It would now be of interest to investigate this possibility and to determine if any relationship exists between "bursting factor" and neuraminidase.

It is advisable to temper the above speculation with the observation that _Cl. welchii_ as a species does not appear to depend for its survival on mechanisms of pathogenicity. With the

exception of food-poisoning strains of *Cl. welchii*, all of the strains examined in the present investigation showed some neuraminidase activity. Some strains were much more active than others in this respect. The general occurrence of such an enzyme system in an essentially commensal or saprophytic organism suggests that the primary rôle of neuraminidase in the life processes of *Cl. welchii* is nutritional and has evolved as a factor of advantage to an organism concerned with the decomposition of dead animal tissue containing neuraminic acid complexes. These observations, however, require little amendment to be applied with equal truth to the lecithinase of *Cl. welchii*, and this enzyme certainly seems to be involved when the organism, albeit inadvertently, assumes its pathogenic rôle in man.

In the further elucidation of the inter-relationships of the diffusible products of *Cl. welchii*, more precise fractionation and serological methods will necessarily play important parts. The solution of the problems initially encountered in the present study has required much more time and energy than originally anticipated, and it has been necessary to develop techniques and investigate many incidental problems concurrently; insurance against misinterpretation of phenomena observed in these complex systems

demanded a comparative method involving multiple tests. Some of the most helpful findings are those which have revealed possible sources of error in the serological approach to this subject. These include the occurrence and nature of non-specific inhibitors of the <u>Cl. welchii</u> haemagglutinin in culture media and sera, the accessibility of serum protein-bound sialic acid complexes to the neuraminidase, the occurrence of a temperature-independent myxovirus receptor-inactivating system, and the presence of two transforming mechanisms in cultures of <u>Cl. welchii</u> which make the interpretation of serum haemagglutination-inhibition tests extremely difficult. Meanwhile, specialists in other disciplines have developed procedures which may be used to confirm some of the speculations based on the present findings. Cassidy, Jourdian and Roseman (1962) have now published a preliminary report on the purification and properties of the <u>Cl. welchii</u> neuraminidase. It should therefore soon be possible to test the above theories using purified products and employing gel diffusion systems and immunoelectrophoresis. Adequate information may then be available to indicate the probable value of neutralisation tests in tissue preparations and in the experimental animal.

Foreknowledge of the position of several pitfalls in a path is in part a deterrent to explore further but may be accepted as a challenge to proceed.

The hypothesis developed in the latter part of this discussion will be tested bearing in mind the caution and encouragement of Miles (1955):

> 'The variety of biochemical structures in nature is not vast enough to warrant our reading a deep significance into every correspondence between a parasite enzyme and a host substrate, or between a host enzyme and a parasite substrate.
>
> . . . . . To be aware of a fallacy, however, does not mean that we should be afraid of manoeuvring ourselves into positions where we might commit it.'

SECTION V : Summary

SUMMARY

1. The literature concerning direct bacterial haemagglutination is reviewed with particular reference to diffusible bacterial haemagglutinins.

2. Theories regarding the nature and function of diffusible bacterial haemagglutinins are developed.

3. Wickham's hypothesis that the *Clostridium welchii* haemagglutinin is identical with the neuraminidase and the enzyme (A-enzyme) which destroys blood group-A substance is summarised.

4. The literature is reviewed relating to: (i) blood group substances, their detection and decomposition; (ii) receptor-destroying enzyme (neuraminidase), its characteristics and its production by various micro-organisms; (iii) haemagglutination by the myxoviruses and its inhibition by mucoproteins containing neuraminic acid residues; (iv) neuraminic acid, its structure and occurrence; (v) the biochemical determination of neuraminidase activity; and (vi) panagglutination, the Thomsen-Friedenreich phenomenon, its relationship to neuraminidase activity, and other mechanisms of inducing panagglutin-

ability of red cells.

5. Probable sources of error in the interpretation of experiments involving direct bacterial haemagglutinins are considered.
6. Methods for the demonstration of the Cl. welchii haemagglutinin are investigated. Titration of the haemagglutinin in tube tests yielded the most informative and most consistent results.
7. The Cl. welchii haemagglutinin is characterised in detail. It possesses affinity for a wide range of red cells and is active over a wide range of pH (5.5 - 9.4) and temperature (4° - 37°C.). It is thermolabile in 30 - 45 min. at 55°C., and is most rapidly inactivated at an acid pH.
8. Agglutination of normal human red cells by the haemagglutinin is most evident in tests at pH 6.8 and 20°C. Removal of the myxovirus receptors of human red cells with Vibrio cholerae neuraminidase renders the cells exceptionally sensitive to the Cl. welchii haemagglutinin.
9. The haemagglutinin is detectable in the supernatant fluid and in the bacillary deposit of centrifuged broth cultures; large amounts are liberated from washed

bacilli by ultrasonic disintegration. The haemagglutinin is thus partly diffusible and partly cell-bound.

10. The cell-bound haemagglutinin is not a mechanism of bacillary adhesion to animal tissue cells but, after liberation in the form of multivalent particles, it becomes adsorbed to the surfaces of red cells and alters their normal mutually repellent character. The haemagglutinin is not spontaneously eluted after adsorption to the red cell surface.
11. A heat-stable inhibitor (C) of the haemagglutinin has been demonstrated in cooked-meat broth and differentiated from the inhibitor (A) of blood group-A isoagglutination, blood group-A substance, which occurs in commercial peptone.
12. Myxovirus haemagglutination is also inhibited by inhibitor C which can be inactivated by incubation with V. cholerae filtrate containing neuraminidase. Inhibitors C and A can be inactivated by Cl. welchii cultures, but the activities against the two inhibitors are not correlated and the ability of a strain to destroy inhibitor C is unrelated to its capacity for haemagglutinin production.

13. There is a general relationship between ability of a strain to produce haemagglutinin and ability to destroy the blood group-A component of peptone, but the results clearly demonstrate that the _Cl. welchii_ haemagglutinin is not the same as the enzyme destroying blood group-A substance.
14. The presence of a pH-sensitive, temperature-independent myxovirus receptor-inactivating system is demonstrated in _Cl. welchii_ cultures. The factor responsible is not the lecithinase or the haemagglutinin. Although the receptor-inactivating agent behaves in many ways like the neuraminidase, it does not appear to be identical with active neuraminidase.
15. A biochemical test for the direct measurement of neuraminidase using egg-white as substrate has been developed. The enzyme of _Cl. welchii_ which splits sialic acid from egg-white is shown to have the characteristics of the neuraminidase.
16. No sialic acid is detectable by normal methods in _Cl. welchii_ cells. Traces of sialic acid are detected in Ellner medium and nutrient broth and a significant amount in cooked-meat broth.
17. The characteristics of the neuraminidase of

Cl. welchii differ from those of the haemagglutinin. Fractionation procedures confirm that the neuraminidase and the haemagglutinin are not identical but seem to be related; both are different from the enzyme destroying blood group-A substance.

18. The absorption of haemagglutinin, receptor-inactivating agent, neuraminidase and lecithinase by human red cells is studied. The haemagglutinin is most readily absorbed but it does not appear to interfere with lecithinase activity.

19. The haemagglutinin can be inhibited specifically by *Cl. welchii* antiserum and non-specifically by inhibitors which occur in saliva, egg-white and serum. The neuraminidase can be inhibited specifically by *Cl. welchii* antiserum; the uninhibited enzyme may attack serum mucoproteins and liberate free sialic acid from normal or heterologous serum.

20. The transforming principle of *Cl. welchii* responsible for the enzymatic induction of panagglutinability of red cells is almost certainly the neuraminidase. A second transforming system is detected in haemagglutinating cultures and this involves a

temperature-independent adsorption mechanism. The second transforming agent may be the haemagglutinin and there is some evidence that it may interfere with access of neuraminidase to its substrate at the red cell surface.

21. The haemagglutinin does not appear to be a naturally occurring product of Cl. welchii. It was not produced by any of more than 100 freshly isolated strains. Good yields are obtained from 48-hr blood agar cultures of laboratory stock strains which have been subcultured for many months; best yields of diffusible haemagglutinin are obtained from sporing cultures in Ellner's medium. The haemagglutinin may be an aggregated or degraded form of neuraminidase which possesses multivalent affinity for its substrate but has lost the ability to complete its enzymatic activity at the red cell surface.

22. The results of the present investigation are discussed. They support the opinion that the haemagglutinin is essentially diffusible, but also provide an explanation for the earlier report that the Cl. welchii haemagglutinin is cell-bound. The results are not consistent with the unitarian hypothesis

that the haemagglutinin, neuraminidase and A-enzyme are identical. Neither haemagglutinin nor neuraminidase is produced by typical food-poisoning strains of *Cl. welchii*.

23. The significance of the findings concerning panagglutination and the Thomsen-Friedenreich phenomenon is discussed. These merit further investigation in view of the present confusion in the literature.

24. The rôle of neuraminidase in the host-parasite relationships of *Cl. welchii* is considered. The enzyme does not appear to be essential to the life processes of the organism *in vitro*. It is not lethal on intravenous injection into mice.

25. The literature concerning the relationship of the lecithinase of *Cl. welchii* to the profound toxaemia generally associated with wound infections caused by this organism is discussed with reference to the theory that an additional toxic factor may be involved.

26. A reaction to the sustained absorption of antigenically altered tissue products produced by neuraminidase activity is considered as a possible component in the mechanism of shock associated with severe

Cl. welchii infections.

27. The probable participation of neuraminidase as an aggressive factor attacking protective mucoproteins of cell-surfaces and constituents of ground substance, and rendering substrate at those sites more vulnerable to lecithinase, is envisaged. The hypothesis is submitted as worthy of investigation in the further development of this work.

SECTION VI : References

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