HAEMOPHILUS INFLUENZAE-LIKE BACILLI IN THE HUMAN VAGINA

P.N.Edmunds, M.B.,Ch.B.,B.Sc.

Bacteriological Unit at the Astley Ainslie
Hospital, Edinburgh.

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C H A P T E R I.

INTRODUCTION AND REVIEW OF LITERATURE

CHAPTER I. INTRODUCTION AND REVIEW OF LITERATURE.

For several years, this laboratory has reported in films and cultures of routine high vaginal swabs, mainly from cases of puerperal pyrexia, small Gram negative bacilli in large numbers, under the name of "Haemophilus influenzae-like bacilli" (to be abbreviated to "HILB" hereafter).

A study of the literature, however, has revealed a striking dearth of information on these organisms. This is surprising, since very large numbers of them have been both seen and cultured regularly in this laboratory and, in fact, they have often been present in almost pure culture. One could only assume that the excessive fastidiousness and slow growth of the organism had led to failure of its isolation on routine media in other laboratories.

Until 1953, few authors had mentioned the occurrence of aerobic Gram negative bacilli in the vagina, with the exception of coliform bacilli and Ducrey's bacillus.

Döderlein (1892) examined 195 pregnant women and divided them into those with normal vaginal secretion and those with pathological secretion. The normal were whitish, thick and very acid, while

the abnormal were only slightly acid or alkaline. A description of the lactobacillus named after him was given, and the acidity of the normal secretion attributed to the action of this bacillus. No mention was, however, made of organisms resembling HILB.

Wegelius (1909) described the flora of the vulva, and showed that at the time of parturition, organisms could ascend from the vulva to the uterus and be demonstrated there for several days. Anderson et al.(1923) studied vulvo-vaginitis in children, but apart from "pseudo-diphtheroids" which were Gram positive, no organism resembling HILB was described by either Wegelius or Anderson et al.

Houlton (1924), in describing the vaginal flora in pregnancy, made the following analysis of "pathological" and "normal" secretions:-

Pathological.

Organisms present.	No. of cases
Streptococci	2
Staphylococci	6
Gram +ve diplococci	10
Gonococci	3
Very short Gram +ve bacilli with rounded ends.	13
Diphtheroid bacilli	2
Gram -ve bacilli	2
B.vaginalis	1

Normal.

Organisms present.	No. of cases.
Staphylococci	3
Gram +ve diplococci	4
Diphtheroid bacilli	1
Very short Gram +ve bacilli with rounded ends.	15
Slender Gram +ve bacilli with spore-like body at one end.	2
Thick Gram +ve bacilli with rounded ends.	1
<u>B.vaginalis</u>	30
Gram -ve bacilli	1

The detection of Gram negative bacilli was reported, in direct films only, in 2 cases with a "pathological" secretion, which failed to grow on culture.

Bryce (1928) in a study of bacterial flora in female genital passages in pregnancy and the puerperium, found unidentified aerobic Gram -ve bacilli in 17 cases of puerperal fever, but the description resembled that of coliform bacilli, not of HILB. Similarly, Pettit and Hitchcock (1933) describing the normal flora of the pre-pubertal vagina found only diphtheroid bacilli, Gram positive cocci and a few Gram negative bacilli which appeared to be mainly <u>Proteus</u> and <u>B.coli</u> species.

Cruickshank and Baird (1929 - 1930) and Cruickshank and Sharman (1934) reported that the vaginal flora in pregnancy could be divided into 3 grades, A (or I), B (or II), C (or III). They

corresponded to 3 pH ranges, as follows:-

Grade A (or I)	Döderlein's bacilli pure, epithelial cells, yeasts + or -.	pH 4.0 - 4.4
Grade B (or II)	Döderlein's bacilli and 1 or more other organisms.	pH 4.6 - 5.6
Grade C (or III)	Heterogeneous: diphtheroids, enterococci, staphylococci, vibrios and rarely coliform bacilli.	pH 5.6 - 7.6

"Vibrios" were mentioned as being present in Group C, i.e. the pH group in which we have found HILB to be most frequently present. Although curved Gram negative forms have been seen occasionally in the present investigation, no cultures have been obtained of anything resembling a vibrio (see Moore, 1954, and other articles mentioned later).

Hardy (1941), in describing the vaginal flora in children, mentioned "diplo-bacilli", "other Gram negative rods" and H.influenzae as being found, the last being rare. The only bacteria he could incriminate in vaginal infection in girls were gonococci, Group A streptococci and "possibly the diplobacillus of Petit" (Diplobacillus liquefaciens), this last being a small Gram negative rod found in conjunctivitis but able to grow on ordinary media, and to liquefy coagulated serum and gelatin.

Henriksen (1947) described 19 strains of Gram negative bacilli from the male and female genitourinary tract. Five were indistinguishable from

H.influenzae, one was a Gram negative coccus arranged like a staphylococcus, and one looked like a short coliform bacillus. The remaining 12 were short plump diplobacilli which grew on ordinary media and were therefore quite different from HILB.

Hart and Brown (1951 and 1952) mentioned the isolation of a small diphtheroid cocco -bacillus in a large proportion of cases of cervicitis. No details were given of this organism.

The first recognisable description of HILB was by Leopold (1953), working in the U.S.A., who stated that the organism grew on a new type of blood agar which the laboratory had just adopted (non-infusion blood agar base of Casman, 1947), with pin-point colonies appearing after 48 hours aerobic incubation. The species of blood used in this medium was not given. It was found in the urine of 53 of 965 males with mild prostatitis, and in 16 of 58 cervical swabs from females with cervicitis. It was also found in the urine of 4 of 9 men whose wives gave positive cultures. Growth could be obtained in thioglycollate broth by the use of heavy inocula and incubation for 48 hours. Illustrations were given showing a micro-aerophilic distribution of growth in this medium, and also the appearance of a 48 hours blood agar culture, showing haemolysis similar to that obtained with HILB.

Acid production was stated to occur from glucose, maltose and dextrin and in some strains from xylose arabinose, using cysteine trypticase agar (Vera, 1948) as the basic medium. Sucrose, lactose, inulin, mannitol and glycerol were not fermented. Nitrates were not reduced and the organism was oxidase negative.

Leopold suggests a close relationship of this organism with the genus Haemophilus but states that the growth requirements, microscopic and macroscopic morphology contra-indicate inclusion in the Mimeae tribe.

The French workers Lutz and Wurch (1954) stated that numerous small Gram negative bacilli not yet identified were seen in many cases where mixed vaginal flora were present. Wurch and Lutz (1955) found in the vaginal flora in 500 cases of leukorrhoea that small Gram negative bacilli were present in 22% of cases. They noted that there were usually few leukocytes but very many of the bacilli, in spite of which, culture on various media remained sterile. In 1956, however, Lutz, Wurch and Grootten reported that the organism grew in 48 hours on 10% blood agar plus 0.3% glucose, giving a small haemolytic colony. Growth was better if yeast extract was added, but factor V from staphylococci failed to enable growth to

occur, even on blood agar (the blood species was not given). The authors noted that "certain elements lightly retained Gram", a fact mentioned also by Brewer et al.(1957) and confirmed in the present investigation. Finally, the organism was stated to be sensitive to penicillin, chloramphenicol, tetracycline, erythromycin, and framycetin, and resistant to streptomycin and neomycin, but only 2 strains were tested. Lutz, Grootten and Wurch (1956), revealed that they used sheep blood in their media, that 10 strains were sensitive to neomycin and that streptomycin sensitivity was variable. Inoculation of a mouse (intra-peritoneal) and a rabbit (intra-dermal) with cultures had no observable effect. The organism was stated to ferment glucose, laevulose, maltose and **starch**, to give variable results with saccharose, xylose and arabinose and to be negative with lactose, galactose, mannite, raffinose, adonite and inosite. The methods used for these tests were not given. The authors named the organism "Hemophilus hemolyticus vaginalis".

Lutz and Burger (1957) gave the incidence for this organism in normal pregnant women as 20% and 28.5% in cases with Trichomonas infection. They suggested that there was an association between the two organisms.

Gardner and Dukes (1954 and 1955), in Texas, described a similar organism under the name "H. vaginalis" and claimed it to be the cause of a newly defined, specific infection previously classified as "non-specific vaginitis". They investigated 602 obstetric and 579 gynaecological cases, and, of the total of 1181, 12% yielded cultures of H.vaginalis (13.3% in gynaecological cases and 10.6% in obstetric). The organism was not found in any of 78 normal control, and 43 miscellaneous control cases, but of 138 cases diagnosed as "bacterial vaginitis", 92% were attributed to H.vaginalis. Their criteria for the diagnosis "bacterial vaginitis" appeared to be leukorrhoea with "a pH of 5.0 or greater in a woman with functioning ovaries and with no trichomonads on wet mount".

Clinical manifestations were given in detail, comparison of H.vaginalis-infected secretion with normal secretions (white and curdy) and trichomonas-infected secretions (offensive, yellow-green, homogeneous) showing the first to be a thin homogeneous film adhering to the vaginal wall, usually of a grey colour and with a disagreeable odour, noticeable to the patient, but less offensive than in the case of trichomoniasis. Frothiness of the discharge was present in 27% of cases of H.vaginalis vaginitis and 34% of cases of trichomoniasis.

On inspection, a minimal degree of redness and oedema of the vulva was noticed in 22% and some evidence of minimal vaginal pathology, as indicated by petechiae or redness, was noted in 11%. There was no obvious relationship between cervicitis and H.vaginalis infection. Apart from the existence of leukorrhoea, local pathology was therefore unconvincing and symptomatology did not appear to be significantly different from that complained of by many patients who were clinically and bacteriologically normal.

Koch's postulates were claimed to have been fulfilled, in that (1) 92% of bacterial vaginitis cases were found to have H.vaginalis infection, (2) the organism was isolated in pure culture in 141 cases, (3) 1 out 13 volunteers who were inoculated with pure cultures developed leukorrhaea and (4) a specimen from this case subsequently yielded the organism in pure culture.

The probable venereal nature of this infection was first suggested by Leopold in 1953 and later by Gardner and Dukes (1955), who recovered the organism by culture from 45 of 47 urethras of husbands whose wives had the "disease". Several husbands who continued to use condoms and whose wives had remained free of infection for 6 months showed positive cultures when finally studied.

This suggested a more or less permanent infection of the male urethra, rather than a mechanical deposition of the organism. Transmission of the infection was also shown, both from husband to wife and from wife to husband.

The authors placed great reliance on the appearance of so-called "clue cells" in the wet mount of leukorrhoea specimens, these being degenerated epithelial cells of a granular appearance, due to crowding of H.vaginalis on their surface. The culture media used were 10% defibrinated sheep's blood agar and Brewer's thioglycollate broth without indicator. Haemolysis on blood agar was a variable feature and colonies were normally detected by reflected light. When haemolysis was present, however, it was a definite aid in the detection of the colonies. Reduced oxygen tension was necessary for isolation on blood agar. The authors stated that citrated human blood was unsuitable for solid medium.

The organism was found, by a disk method, to be sensitive to the tetracycline group, usually resistant to chloramphenicol, and resistant to penicillin, polymyxin and streptomycin. This contrasts strongly with the results of the present investigation. For treatment, therefore, vaginal aureomycin or terramycin was given, only 1 out of 27 cases failing to respond. Oral tetracycline

proved unsatisfactory. Owing to infection of the husband, recurrences tended to ensue after treatment, if the husband was not treated as well. No details were given as to how this was done.

The biochemical reactions given by the organism as described by Gardner and Dukes, were similar to those reported by Leopold (1953) and by Lutz, Wurch and Grootten (1956) except that the last authors said that sucrose fermentation was variable, whereas the other authors gave it as definitely negative. Gardner and Dukes used the same basic medium (cysteine trypticase agar) as Leopold, for fermentation tests.

The only worker in this field to suggest that the organism concerned is in fact H.influenzae itself, was Deming (1955), in a rather vague description of the isolation of this organism from cases of leukorrhoea not caused by trichomonas or monilia. Haemolysis was given among the diagnostic criteria, which is usually absent in true H.influenzae, and no mention of growth factor requirement or the occurrence of satellitism was made. The one case history given suggested a mode of infection by transference of organisms from nasal secretions to the vagina via toilet paper, but no real evidence was given that this had in fact occurred. All the other authors on this subject are agreed that the predominating Gram negative

bacillus concerned in vaginal specimens is not H.influenzae.

Ray and Maugham (1956) reported from Oregon, U.S.A., the results of an investigation into 447 clinic patients, 2/3 of which were gynaecological cases and 1/3 obstetric, together with 74 cases of vaginitis seen in private practice. Of the 447 clinic patients, 68 (15.2%) showed "H.vaginalis", and these constituted 94% of the total of 72 cases which were diagnosed as "bacterial vaginitis", a group which apparently consisted of all cases with "infectious organisms" (not defined) where neither trichomonas nor monilia were found microscopically, and regardless of whether any symptoms of any kind were present. These results were based on microscopic findings only, culture evidently having failed. Of the 68 patients with "H.vaginalis", 19 were asymptomatic and there was no sign of local irritation.

Amies and Jones (1957), in Canada, were able to isolate a similar organism from 5.1% of 371 cervical swabs, using serum yeast agar plates, which gave dew-drop colonies 0.5 - 1.0 mm. in diameter after 24 hours incubation, presumably at 37°C. They found that these colonies could be easily mistaken for faecal streptococcal colonies, a description quite unlike that given by Leopold (1953), Gardner and Dukes (1955) and Lutz et al,

(1956) of pin-point colonies barely visible until 48 hours had passed. In the present investigation it has been found that enterococci produce colonies of quite a different order of size from that of HILB.

Amies and Jones assumed that the action of yeast extract in encouraging growth was due to V factor, and suggested that the failure of Fildes' blood digest to enhance growth might mean that X factor was not required. Their organism was stated not to be able to grow under complete anaerobiosis and, in serum thioglycollate medium, maximum growth occurred at or near the surface.

Fermentation reactions were carried out, using serum yeast agar plus 1% of the test substance, on 23 strains, all of which failed to ferment lactose, dulcitol, mannitol, trehalose and rhamnose. Dextrose was attacked by all but 1 strain. Irregular results were obtained with other carbohydrates, maltose, arabinose, laevulose, xylose, glycerol, raffinose, salicin and sucrose all being fermented by some strains. The authors claim that nitrates were reduced in every case, and that 2 strains were indole positive, but the methods of carrying out these tests were not given. Their strains were all sensitive to tetracycline, chloramphenicol and erythromycin, about half were resistant to penicillin and one was resistant to streptomycin.

Slide agglutination tests showed that 18 strains reacted to 1 antiserum, 5 strains were agglutinated by a second antiserum and the 2 remaining strains were agglutinated by their homologous antisera only. The spontaneous agglutinability noted by us, also occurred on attempted tube agglutination, even when the salt content of the suspension was reduced, phosphate buffer used, or a protective colloid added. This occurred in spite of an apparently smooth colonial appearance.

Amies and Jones failed to produce disease in animals, except for a raised lesion on intra-dermal inoculation of a rabbit of a heavy suspension of living bacilli.

An interesting theory was advanced by these authors that L-forms, which they claimed to be able to produce by the action of penicillin, may in fact be the pleuropneumonia-like organisms (PPLO) of Dienes and many others. This question is dealt with later in this thesis.

Brewer <u>et al</u>.(1957) reported an investigation into 211 gynaecological cases aged 20 - 63, all complaining of leukorrhoea. Of these, 89 showed the presence of small Gram negative bacilli, either by microscopic or cultural means. Casman's agar medium as described by Leopold (1953) was used, 5% defibrinated rabbit blood being added. Smooth "dew-drop" pin-point colonies were regarded as being

typical of H.vaginalis. No mention was made of haemolysis occurring on the blood agar, and pure cultures could not be maintained for more than 2 - 3 sub-cultures. Of the 89 cases with H.vaginalis present, 59 were without trichomonas or candida, 21 showed trichomonas, 6 candida, and 3 both.

Forty-seven of the 59 cases, in which H.vaginalis was not accompanied by the other two pathogens, were analysed according to the severity of the leukorrhoea etc. Leukorrhoea was slight in 40 cases, moderate in 7, not severe in any. The discharge was white or greyish-white in 42, yellow in 3, green in 1 and clear in 1. It had a disagreeable odour in all cases, and the pH averaged 5.2. Vaginitis was claimed to be present in a higher proportion than that reported by previous authors, e.g. Gardner and Dukes (1955). Redness of the vagina was seen in 34 of 47 and absent in 13, usually being diffuse and slight, but in a few cases, punctate. An interesting point is that 16 of the 47 (34%) had strictly vaginal involvement as against cervical, since the cervix had previously been removed by complete hysterectomy. One case had severe vulvitis, with redness, oedema and marked pruritus. H.vaginalis was isolated from secretions scraped from the vulva, but was not recovered from the vagina. Treatment was successfully carried out, using terramycin and polymyxin suppositories, H.vaginalis being promptly eradicated

in 36 out of 40 occasions.

The observation of Gardner and Dukes (1955) that the organism usually appears in pure culture was not borne out in this article, pure cultures being rarely seen by the authors. A further interesting observation was that many transplanted strains became Gram variable, some being definitely Gram positive and like small diphtheroids.

Vibrios in the Vagina:

A group of authors have described vibrio-like organisms in the vagina. Moore (1954) reported the occurrence of anaerobic vibrios in vaginal specimens, seen at first only in wet films. They showed the typical curved shape and exceptionally active motility characteristic of vibrios. Culture failed until the author incubated blood agar plates anaerobically with 10% CO_2 added. They gave colonies 0.7 mm. in diameter after 3 days incubation. Dense greening occurred round each colony. The motility, atmospheric requirements, colonial and microscopic morphology clearly differentiate these organisms from HILB. An interesting feature in common with HILB was the Gram variable reaction, definitely Gram positive forms occurring under certain circumstances, although they were mainly Gram negative.

Previous observations had been made on similar organisms in the vagina, by Curtis (1913),

Cruickshank and Baird (1929-30), Rosebury (1948) and Laughton (1948).

Although the main purpose of this thesis is to deal with HILB, it has become more and more obvious that there is a connection between this organism and PPLO, and some investigation has had to be made into this connection. A short review of the relevant literature on L-forms and PPLO is therefore indicated.

The organism of pleuropneumonia was first recognised and cultured by Nocard and Roux in 1898, the morphology being described by Bordet, (1910) and Borrel <u>et al</u>, (1910), but it was not until many years later that the existence of many other similar organisms was recognised, for example, the organism of contagious agalactia of sheep (Bridré and Donatien, 1923, 1925). Many organisms of this group have since been isolated from the respiratory and genito-urinary tracts of mice, cattle, man, rats and dogs (Nelson, 1936a, b, 1937, 1940; Edward, 1940, 1947; Edward <u>et al</u>, 1947; Dienes and Edsall, 1937; Dienes, 1940; Klieneberger-Nobel, 1945; Beveridge <u>et al</u>, 1946; Salaman <u>et al</u>, 1946; Klieneberger and Steabben, 1937; Woglom and Warren, 1938; Klieneberger, 1938; Preston, 1942; Shoetensack, 1934; Edward and Fitzgerald, 1951).

All these strains require a high proportion of animal protein, e.g. 20-30% serum. Soft agar

(0.5% New Zealand) is better than 1.0% (N.Z.). Boiled blood agar has been used (Dienes, 1940; Klieneberger-Nobel, 1945). Edward (1947) described a selective medium for PPLO containing thallium acetate, which is highly bacteriostatic for aerobic spore-bearing bacilli and Gram negative bacilli. Twenty per cent. horse serum and 10% yeast extract were also included. Certain batches of horse serum proved defective and this was what necessitated the addition of yeast extract. The factor present in this extract was not V factor, as it was resistant to autoclaving at 120°C. for 60 minutes. The factor, when required because of defective serum, could not be replaced by boiled blood or by liver extracts.

Randall _et al_.(1950), in a study of 300 gynaecological hospital admissions, found PPLO in the cervix of 78 (26%). They were found in all age-groups, but more commonly in the 15-40 group, and were more frequently isolated in cases of leukorrhoea, in which the incidence was 55% in moderate and 71% in severe cases. One case of tubo-ovarian abscess gave a pure culture of PPLO from pus evacuated surgically from a closed abscess cavity.

Klieneberger-Nobel (1950), in a study of methods used for demonstrating the cytology of PPLO, showed that the slightest distortion during processing ruined the true morphology of the organism. This was confirmed by other workers,

including Edward (1954) who stated that the most reliable method for identification of PPLO in culture, was to examine the intact colony with a dissecting microscope using obliquely transmitted light, thus demonstrating the characteristic umbonate centre and the less opaque halo of growth around it.

In 1953 and 1954, Edward showed that there was a difference in growth requiremeht of PPLO and L-forms of bacteria, PPLO requiring cholesterol while L-forms were able to grow without it, when a medium was used in which serum was replaced by albumin plus the acetone-insoluble fraction of egg yolk. Edward (1954) stated that some PPLO ferment carbohydrate while others lack this power. All the positive strains tended to form acid from the same carbohydrates, namely, glucose, maltose, dextrin, starch and glycogen, some also from fructose, galactose and mannose. Lactose, saccharose, mannite and dulcite were never fermented. This contrasted with the finding that L-forms fermented the same carbohydrates as their parent bacterium. Finally, unlike bacteria in general, PPLO were prevented from growing by the presence of specific antiserum.

Edward considered that PPLO should be placed in a class apart from bacteria rather than in a new

genus or family of bacteria. The same author considered that, although found more frequently in the genital tract when inflammation was present, they probably did not initiate genital infection. This view was confirmed by Klieneberger-Nobel (1954), who said that PPLO need a second factor, such as a breakdown in the defence mechanism of the host, an adjuvant, or another infective agent, before being able to cause disease in a susceptible host. The author, although the initiator of the term "L" as a label for PPLO, said in this article that important differences, e.g. in morphology, existed between L-forms and PPLO. There was no evidence to confirm the suggestion that PPLO had been derived from bacteria and the group was to be regarded as a distinct class of micro-organism intermediate between bacteria and viruses.

L-forms were first reported by Klieneberger (1935) who found that a pleuropneumonia-like organism, now referred to as L1, occurred in cultures of Streptobacillus moniliformis, living apparently in symbiosis with the bacilli. Later, the author considered, in company with other workers (Dienes, 1942; Heilman, 1941; Brown and Nunemaker, 1942; and Smith, 1941) that L1 was merely a variant of the bacillus (Klieneberger-

Nobel, 1949).

L-forms, which have a marked similarity in morphology to PPLO, have been isolated now from numerous bacteria, e.g. Proteus (Dienes, 1949), B.coli (von Prittwitz-Gaffron, 1956), Bacteroides (Dienes, 1948), F.necrophorus (Klieneberger-Nobel, 1949), typhoid bacilli, Salmonellae and Shigella (Dienes, 1948; Dienes et al, 1950; Weinberger et al, 1950), and Clostridia (Dienes, 1950). Gram positive spore-bearers and H.influenzae are also included in the list by Wilson and Miles (1955).

The methods of producing L-forms have included penicillin (Dienes, 1949, and others), exposure to cold, 3% NaCl, 0.1% LiCl, (Klieneberger-Nobel, 1949), 4% glycine, antibody plus complement, and bacteriophage (Dienes et al, 1950).

When the stimulus of the agent is removed, L-forms generally revert back to the bacillary forms, especially when grown in fluid medium. This compares with the tendency mentioned below for PPLO cultures to produce diphtheroid bacillary forms in fluid medium. L-forms as well as PPLO are always resistant to penicillin, and also more resistant to the L-forming agents listed above, than are the parent bacilli. Some L-forms require anaerobic conditions for growth, e.g. Salmonella L-forms (Weinberger et al, 1950). Most PPLO grow better

aerobically, but there are exceptions (Wilson and Miles, 1955). However, Edward (1947) stated that PPLO from the human genital tract grew equally well aerobically, anaerobically or in 10% CO_2.

A most interesting and suggestive development in the last few years is the reporting, by the following authors, of apparent conversion of PPLO to diphtheroid bacilli.

Minck (1953) reported that 5 strains of PPLO isolated from the genital tract of women by repeated picking of single colonies have changed into Gram positive bacilli resembling diphtheroids. It was not possible to separate these 2 forms, but the PPLO predominated in solid media, the diphtheroids in liquid. Both forms had the same fermentation properties and a general similarity in antigenic properties. By phase-contrast microscopy, the author demonstrated transformation of the bacillary forms into L-forms in the absence of antibiotic, and in one case, transformation of L-forms into bacilli.

Peoples _et al_.(1955) noted that when the Campo strain of PPLO was grown in large volumes of broth, minute colonies, consisting of bacilli resembling Corynebacteria, grew in the broth, and could be transferred with a needle. They were inert biochemically. Statistical analysis was claimed to show that the chance of these having arisen as con-

taminants was less than 1 in 10,000. The same authors (Smith et al., 1957) showed that these diphtheroid bacilli did not ferment carbohydrates, whereas diphtheroids isolated from the air,and the oral cavities of the investigators, did. Serological examination showed that the strains of PPLO were related, and that there was cross-agglutination between the derived diphtheroids and the PPLO. No cross-reaction was found between the derived diphtheroids and strains of PPLO which did not produce diphtheroid forms. Attempts at conversion of the derived diphtheroids back to the PPLO forms were not successful.

Wittler et al.(1956) grew PPLO in Hela cell tissue culture, using added yeast extract and mucin. They claimed that conversion of this organism to a corynebacterium resulted.

CHAPTER II

INCIDENCE OF H. INFLUENZAE-LIKE BACILLI IN VARIOUS CLINICAL GROUPS, AND THEIR RELATION TO OTHER VAGINAL FLORA.

CHAPTER II. INCIDENCE OF HILB IN VARIOUS CLINICAL GROUPS AND THEIR RELATION TO OTHER VAGINAL FLORA.

Section 1. Materials and Methods.

A. Collection of specimens.

Through the co-operation of the clinicians concerned, 2 well-soaked high vaginal swabs and 2 smears were obtained from 276 out-patients and in-patients in Bruntsfield and Elsie Inglis Hospitals. In addition, there were 3 cases of vaginal discharge in very young girls (aged 2½ - 3½) seen at the Out-Patients' Department of the Royal Hospital for Sick Children, Edinburgh.

Thirty-nine cases had to be dropped from the series, due to treatment having been given before the specimen was taken, gross contamination of cultures having occurred, or later investigation having showed that they did not fit into the groups being studied.

The 240 cases thus remaining were divided up as follows:-

Ante-natal group: (hereafter abbreviated to AN).

Forty-four normal pregnant women attending the ante-natal clinic at the Elsie Inglis Maternity Hospital, in March-April 1957. These were taken at random and consisted mainly of 2½ - 5½ month pregnancies. None of the women had more than a slight vaginal discharge, 2 patients with a

definite discharge having been excluded from the group.

Puerperal pyrexia group: (hereafter abbreviated to PP).

Forty-five in-patients whose temperature rose to 99°F. or over during the first week after delivery, without any obvious explanation such as breast abscess, urinary infection or venous thrombosis.

Puerperal control group: (hereafter abbreviated to PC).

Twenty-six normal puerperal women, in the same hospital as the PP group, whose temperature remained below 99° F. during the first week after delivery. We were unable to obtain specimens from more than this number, owing to the natural disinclination of nursing and medical staff, not to mention the patients, for taking the requisite specimens without any clinical indication for it.

Gynaecological Group: (hereafter abbreviated to G).

Forty-two women of reproductive age, who attended Out-Patient clinics at Bruntsfield Hospital, from March to June 1957, suffering from leukorrhoea, vaginitis and/or cervical erosion or cervicitis.

Gynaecological control group: (hereafter abbreviated to GC).

Forty-two cases in the same category as the

above group, except that they suffered from some condition other than those indicated, usually pelvic floor weakness.

Post-menopausal group: (hereafter abbreviated to PM).

It was considered necessary to separate these cases from the G group, in view of the fundamental change in the normal vaginal secretion and bacterial flora at the menopause. The 18 women in this group had leukorrhoea, vaginitis and/or cervical erosion or cervicitis.

Post-menopausal control group: (hereafter abbreviated to PMC).

Fourteen cases, the same as above except that they suffered from conditions other than those just mentioned, mostly pelvic floor weakness.

Miscellaneous cases:

Six post-natal women with leukorrhoea, seen 6 weeks after delivery, as out-patients at the Elsie Inglis Hospital, and 3 cases of vaginal discharge in very young girls, already mentioned.

B. Laboratory methods used in examining specimens.

Measurement of pH:

Accurate estimation of pH of vaginal secretions was not attempted. Tests were carried out to determine whether the material of the swab affected the pH of the secretions, as follows.

Samples of 5% human plasma in tap-water were

adjusted with N/10 HCl, using a B.D.H. capillator, to pH 5.6 and pH 4.4. An unused swab on the usual type of wooden applicator was soaked in the fluid in each tube and the pH measured immediately and after 1 hour at room temperature. At both pH levels, no change occurred. A similar technique, but without the hour's wait, was used at pH levels 7.3 and 6.6 and again no change was produced by the action of the swab. The buffering effect of vaginal secretion was thought likely to be more pronounced than that of 5% plasma water and so, even less likely to be affected by being soaked into the swab during transport.

It was considered therefore that the purposes of this study would be sufficiently served by measuring the pH of the vaginal secretion after expressing it from the specimen swab into 0.5 ml. of sterile tap-water (which was neutral in reaction). This was done with a B.D.H. capillator, and the result was considered to be accurate to within 0.2 pH units.

Microscopic Examination:

One smear was fixed by heat and stained Gram, alcohol being used for de-colourising, and being left in contact with the smear for 45 seconds, with occasional renewal. The other smear was stained by Leishman's method. Diagnosis of flora type,

cell contents, and trichomonas and yeast infection, was made by examination of these slides. We have followed the example of Liston and Liston (1939) in using Leishman-stained smears for the diagnosis of trichomonas, as we have found this to be a reliable method once the necessary skill has been attained in recognising the rather distorted appearance of the organism in such smears. The proportion of positive diagnoses made by this method in our hands, compares favourably, at 25 - 30% of routine leukorrhoea smears, with that obtained by other workers, using more conventional methods (see Whittington, 1957).

For diagnosing the presence of HILB, reliance was placed on culture rather than on film appearance, although it was usually possible to tell in advance from seeing numerous small Gram negative bacilli that this organism would be isolated.

<u>Culture Technique:</u>

The main medium used was digest blood agar, prepared as described in the Appendix (No.4). Media for isolation of PPLO included thallium acetate soft blood agar, plasma thallium acetate agar plates, and thallium acetate sloppy agar, as described in detail in the Appendix (numbers 58, 59 and 60 respectively).

One of the 2 vaginal swabs received from each patient was cultured aerobically on blood agar, McConkey agar and PPLO medium (see below). The other swab, after mixing with sterile tap-water for pH estimation, was cultured in Robertson's cooked meat medium and on a blood agar plate which was incubated anaerobically in a McIntosh and Fildes jar. The cooked meat medium was used alone for primary anaerobic culture for the first 75 specimens, but thereafter reliance was mainly placed on the anaerobic blood agar plate, the Robertson's medium then being kept in reserve in case of contamination of the plate. HILB were then recognised by their minute haemolytic colonies and confirmed by filming. Only those which were definitely Gram negative were included, although in the original smear and later in fluid cultures, a tendency to be Gram positive was noted in some strains.

The technique for isolating PPLO was varied during the series, as experience was gained. For the first 57 specimens thallium acetate soft blood agar (Appendix number 58) was used. This was then replaced by plasma thallium acetate agar (No.59). Identification of PPLO colonies on this medium was greatly facilitated by the incorporation of a sterile microscope slide in the plate of soft agar.

This gave a thin layer of agar over the slide in the centre, with good illumination when the plate was viewed under the low power of the microscope by oblique transmitted light. The refraction caused by the edge of the slide was particularly useful in showing up the characteristic morphology of any colonies that happened to lie above this edge. It was found unnecessary to make special stained preparations by impression or other techniques, as the microscopic colony appearance was diagnostic (v. Figs 1,2). This is also the method recommended by Edward (1954) as being the most reliable.

PPLO were found to grow surprisingly well on the digest blood agar used for HILB and other organisms, and often survived on this medium when accompanying HILB had died out.

After the 108th specimen, it was found better to use sloppy thallium acetate agar (No. 60) for primary culture, with sub-culture after 48 hours incubation on to the plasma thallium acetate agar plates.

Plates were incubated 48 - 72 hours at 37° C. Single colonies were then picked, with or without the aid of a hand lens, of HILB and PPLO. Two to four colonies of HILB were sub-cultured on each of 2 duplicate blood agar plates, and, in addition, a

heavy inoculum obtained by sweeping the loop through the general growth of HILB, was plated out in duplicate. If the single colonies failed to yield a growth on the first picking, it was usually possible to obtain a successful sub-culture from single colonies on the secondary plate after 2 - 3 days further incubation. As can be readily appreciated, the technique required for the isolation and purification of HILB is most difficult and time-consuming. It requires the most meticulous care and patience, with numerous disappointments lying in wait for the researcher. Contamination of plates is a continual bugbear, due to the necessity for thick moist plates and prolonged incubation of a very rich medium.

Once pure cultures were obtained (and this sometimes required repeated pickings of single colonies to separate PPLO from HILB) sub-cultures were made every 48 hours, each strain being freeze-dried as soon as possible by the technique described in the Appendix, and stored in vacuo at 4° C. Cultures were in this way kept viable for many months.

Records of results:

The original specimen was received along with a special duplicated form in which name, age, date, address, name of doctor, clinical group, presumptive diagnosis, temperature and a list of genito-

urinary and other symptoms were requested. The form and specimen were given the "T" number of the series. Later on, after the diagnosis had been confirmed, numerous visits were made to the hospitals concerned, to study case-notes in order to confirm or amplify any statements on the original form and to make sure that treatment had not been started before the specimen was taken. Many cases had to be cancelled for this last reason. Temperature charts were checked also for height and duration of fever.

In the laboratory, original observations on each case were recorded on the back of the form and were transferred to a notebook, in order of serial T numbers.

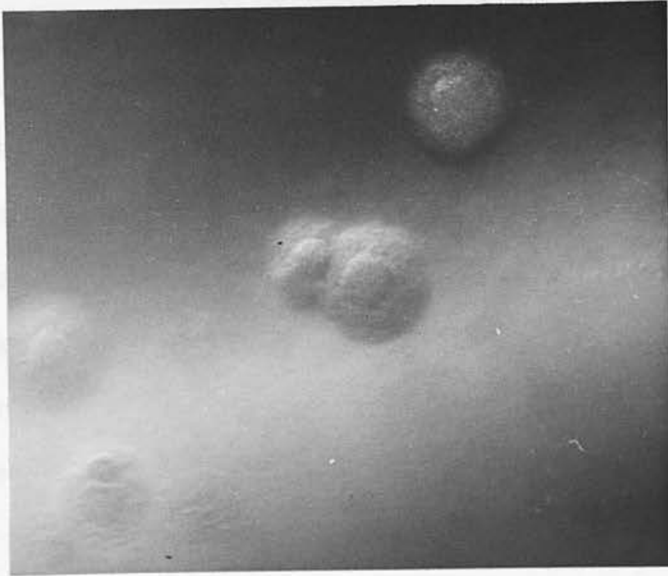

Fig. 1. PPLO colonies on thallium acetate agar, with edge of incorporated slide showing faintly. Culture incubated 4 days at 37°C. X 100.

Fig. 2. PPLO colony on thallium acetate agar, showing typical morphology, with compact central zone and diffuse peripheral zone. The light band is the edge of the slide incorporated in the medium. X 100

Section 2. Results:

The main protocol is seen in Table 1 which represents the original observations, with omission of names, addresses and clinical notes, while the order has been arranged so that the specimens are in their own clinical group, as defined above.

The following numbers had to be cancelled because of treatment having been given before the specimen was taken, because of gross contamination of cultures, or because there was some obvious cause for pyrexia other than HILB infection.

T numbers 2, 5, 6, 9, 40, 97, 134, 138, 149, 154, 171, 172, 175, 189, 198, 213, 241, 242, 244, 246, 247, 248, 257, 258, 260, 261, 263, 268, 273, 276, 278, 279; total 32.

A further 7 were excluded from the main groups as they did not fit into these.

T114, doubtful whether GC or PMC.

T222, category not known.

T.78 and T82, leukorrhoea during ante-natal period.

T161, normal post-natal.

T215, vaginal discharge during labour.

T235, vaginal discharge during puerperium.

These 7 were available for analysis of results as regards the relation of HILB to other pathogens, to PPLO, to pH, and to type of vaginal flora.

TABLE 1.

Protocol of results, including pH, cells and bacteria present, flora type, serial number and clinical group.

Clinical Group	pH.	Trichomonas	Monilia or other yeasts	HHLB	PPLO	Polymorphs	Epithelial cells	Diphtheroid bacilli	Anaerobic non-sporing Gram + bacilli	Coliform bacilli	H.influenzae	Anaerobic Gram- bacilli	Staph.aureus	Staph.albus	Non-haemolytic streptococci	Strep.viridans	Strep.faecalis	Strep.pyogenes	Anaerobic streptococci	Cl.welchii	Subtilis group	N.gonorrhoeae	Flora Type	Serial T. number
	6.6	-	-	-	-	+++	+++	-	-	-	-	-	-	✓	-	-	✓	-	-	-	-	-	?	1
	5.8	-	-	+++	-	-	-	++	-	+	-	-	-	++	✓	-	-	-	-	-	-	-	III	7
	6.8	-	-	+	-	+++	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	II	29
	6.2	-	-	+++	-	+++	+++	✓	-	-	-	-	-	++	-	-	-	-	-	-	✓	-	III	30
	6.4	-	-	-	✓	+++	+++	✓	-	-	-	-	-	-	-	✓	-	-	-	-	-	-	III	65
	6.4	-	-	+++	-	++	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	III	66
	6.8	-	-	+++	-	-	-	-	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	III	87
	6.6	-	-	+++	-	+++	+++	-	-	-	-	-	-	✓	-	-	✓	-	-	-	-	-	III	88
	7.2	-	-	-	-	++	++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	?	107
	6.6	-	-	-	-	-	+++	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	?	133
	6.6	-	-	+++	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	?	144
	6.8	-	-	++	-	+++	+++	-	-	+	-	-	-	++	-	-	-	-	-	-	-	-	?	145
	6.6	-	-	+++	-	++	++	+++	-	-	-	++	-	++	-	-	-	-	-	-	-	-	III	146
	6.2	-	-	+++	+++	++	+++	++	-	-	-	-	-	+	-	-	-	-	✓	-	-	-	III	167
PP	6.8	-	-	+	+++	+++	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	III	168
	7.0	-	-	++	-	++	++	-	-	-	-	-	-	+	-	-	-	-	✓	-	-	-	?	169
	6.8	-	-	+++	-	-	+++	+	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	III	182
	6.8	-	-	+++	-	+	++	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	III	183
	6.8	-	-	++	+++	++	++	-	-	-	-	-	-	+	-	-	-	-	++	-	-	-	III	194
	6.8	-	-	+++	-	+++	+++	-	-	++	-	++	-	+	-	-	-	-	-	-	-	-	III	214
	6.2	-	-	+++	+++	+++	-	+	-	-	-	-	+	++	-	-	-	-	++	-	-	-	III	216
	6.8	-	-	+++ ⊙	-	+++	++	-	-	-	-	-	-	++	-	-	++	-	-	-	-	-	III	217
	6.6	-	-	- ⊙	-	+	-	+	-	-	-	-	++	+++	-	-	-	-	-	-	-	-	?	218
	6.8	-	-	+++	-	-	+	-	-	++	-	-	-	++	++	-	++	-	-	-	-	-	?	220
	6.4	-	-	+++ ⊙	-	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	?	224
	6.8	-	-	- ⊙	-	+++	+++	-	-	-	-	-	-	++	-	-	-	-	+++	-	-	-	III	234
	•	-	-	+++	-	•	•	+	-	-	-	-	-	-	-	-	-	-	++	-	-	-	?	236
	6.6	-	-	+++	+	+++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	?	237
	6.8	-	-	-	✓	-	-	+	-	++	-	-	-	-	-	-	+	-	+++	-	-	-	III	239

TABLE 1. (contd.).

Clinical Group	pH.	Trichomonas	Monilia or other yeasts	HILB	PPLO	Polymorphs	Epithelial cells	Diphtheroid bacilli	Anaerobic non-sporing Gram + bacilli	Coliform bacilli	H.influenzae	Anaerobic Gram - bacilli	Staph.aureus	Staph.albus	Non-haemolytic streptococci	Strep.viridans	Strep.faecalis	Strep.pyogenes	Anaerobic streptococci	Cl.welchii	Subtilis group	N.gonorrhoeae	Flora Type	Serial T. number
PP	6.6	-	-	+++	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	?	243
	6.6	-	-	++	+++	+++	++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	III	251
	6.6	-	-	-	-	++	+	-	-	+	-	-	-	+++	-	-	-	-	-	-	-	-	III	252
	6.0	-	-	+++	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	III	253
	.	-	-	- ⊙	-	+++	++	-	-	-	-	-	-	-	-	-	-	-	+++	-	-	-	III	255
	.	-	-	-	-	+++	+	-	-	-	-	-	+	+	-	-	-	-	++	-	-	-	?	256
	.	-	-	+++	-	++	++	-	-	-	-	-	-	+	++	-	-	-	-	-	-	-	III	262
	.	-	-	+++ ⊙	-	++	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	III	264
	.	-	-	+++ ⊙	-	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	III	265
	.	-	-	+++ ⊙	✓	++	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	III	266
	.	++	-	-	-	+++	+++	-	-	-	-	-	-	+	++	-	-	-	+++	-	-	-	III	267
	.	-	-	+++	-	+++	++	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	III	269
	.	-	-	-	-	-	++	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	?	272
	.	-	-	++	+++	-	++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	III	274
	.	-	-	+++	-	++	+++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	III	275
	.	-	-	-	✓	+++	+	+++	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	III	277
PC	6.6	-	-	-	-	-	++	+	✓	-	-	-	-	+	-	-	-	-	-	-	-	-	?	3
	6.2	-	-	-	-	++	++	-	-	+	-	-	++	++	++	-	++	-	-	-	-	-	III	4
	6.2	-	-	-	-	-	+	+++	-	-	-	-	-	+	-	-	-	-	-	-	-	-	?	16
	6.8	-	-	-	-	-	+	++	-	-	-	-	-	+	-	-	-	-	-	-	-	-	III	17
	6.6	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	III	18
	7.0	-	-	+++	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	?	34
	7.2	-	-	-	-	-	+	-	-	-	-	-	-	+	-	++	-	-	-	-	-	-	?	36
	6.6	-	-	+++	+++	+	+	-	-	-	-	-	-	✓	-	-	-	-	-	-	-	-	III	38
	7.2	-	-	-	-	-	+	-	-	-	-	-	-	++	-	-	+	-	-	-	-	-	III	54
	6.6	-	-	-	-	++	++	-	-	+	-	-	-	-	-	-	✓	-	-	-	-	-	?	63
	6.4	-	-	-	-	++	++	+++	-	-	-	-	-	+	-	-	-	-	-	-	-	-	III	64
	7.2	-	-	-	-	-	+++	-	-	-	-	-	-	+	-	++	-	-	✓	-	-	-	III	85
	7.2	-	-	✓	-	-	+	-	-	✓	-	-	-	-	✓	-	-	-	-	-	-	-	?	86
	6.6	-	++	-	-	-	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	?	89
	7.2	-	-	-	-	++	++	++	-	-	-	-	-	+	-	+	-	-	-	-	-	-	?	96
	7.3	-	-	-	-	++	+++	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	?	108
	7.0	-	-	+	-	++	++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	?	109
	6.8	-	-	-	-	++	++	-	-	-	-	-	-	-	-	-	-	-	✓	-	-	-	III	110

TABLE 1.(contd.).

Clinical Group	pH	Trichomonas	Monilia or other yeasts	HILB	PPLO	Polymorphs	Epithelial cells	Diphtheroid bacilli	Anaerobic non-sporing Gram + bacilli	Coliform bacilli	H.influenzae	Anaerobic Gram - bacilli	Staph.aureus	Staph.albus	Non-haemolytic streptococci	Strep.viridans	Strep.faecalis	Strep.pyogenes	Anaerobic streptococci	Cl.welchii	Subtilis group	N.gonorrhoeae	Flora type	Serial T. number
PC	7.2	-	-	+++	-	-	+	++	•	-	-	-	-	++	-	-	-	-	-	-	-	-	?	170
	6.2	-	-	- ⊙	-	+++	++	+	-	-	-	-	-	++	-	-	-	-	++	-	-	-	III	205
	6.6	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	?	212
	7.0	-	-	+++	-	-	+	-	-	-	-	+	-	++	++	-	-	-	-	-	-	-	?	219
	7.0	-	-	++	-	+	+	+	-	-	-	-	-	++	-	-	-	-	-	-	-	-	?	221
	6.8	-	-	+++	+++	++	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	III	238
	•	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	++	-	-	-	?	270
	•	-	-	- ⊙	-	-	+	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	?	271
AN	4.8	-	-	-	-	-	++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	I	19
	4.8	-	-	++	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	I	20
	5.0	-	++	-	-	-	+	++	-	-	-	-	-	++	-	-	-	-	-	-	-	-	?	21
	7.0	-	-	+++	-	-	+	-	-	-	-	-	-	+	-	-	++	-	-	-	-	-	II	22
	4.6	-	-	-	-	-	+	-	-	+	-	-	-	-	-	++	-	-	-	-	-	-	I	23
	5.2	-	++	-	-	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	24
	5.6	-	++	-	-	+++	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	I	25
	4.8	-	++	-	-	+++	+++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	I	26
	4.6	-	+++	-	-	++	+++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	I	27
	4.6	-	-	-	-	-	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	I	28
	4.4	-	-	-	-	-	+++	+++	✓	-	-	-	-	-	-	✓	-	-	-	-	-	-	I	45
	5.0	-	-	+++	-	-	+++	-	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	III	46
	4.4	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	47
	4.8	-	-	-	++	-	+++	++	-	-	-	-	-	++	-	-	-	-	-	-	-	-	III	48
	4.8	-	-	-	-	-	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	I	49
	4.4.	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	50
	4.8	-	-	-	-	++	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	I	51
	4.8	-	+++	-	-	+++	+++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	II	52
	4.4	-	-	-	-	++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	53
	4.6	-	-	-	-	-	+++	-	-	✓	-	-	-	++	-	-	✓	-	-	-	-	-	II	67
	4.2	-	-	-	-	-	+++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	68
	4.4	-	-	-	-	-	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	69
	4.4	-	-	-	-	+++	+++	-	-	-	-	-	-	✓	-	-	-	-	-	-	-	-	I	70
	4.2	-	-	-	-	++	+++	-	-	-	-	✓	-	++	-	-	-	-	-	-	-	-	I	71
	5.2	-	++	-	-	+++	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	II	72
	5.2	-	-	+++	-	+	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	73

TABLE 1.(contd.).

Clinical Group	pH.	Trichomonas	Monilia or other yeasts	HILB	PPLO	Polymorphs	Epithelial cells	Diphtheroids	Anaerobic non-sporing Gram + bacilli	Coliform bacilli	H.influenzae	Anaerobic Gram - bacilli	Staph.aureus	Staph.albus	Non-haemolytic streptococci	Strep.viridans	Strep.faecalis	Strep.pyogenes	Anaerobic streptococci	Cl.welchii	B.subtilis group	N.gonorrhoeae	Flora Type	Serial T. number
AN	4.4	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	74
	5.4	-	-	+++	-	-	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	III	75
	5.0	-	++	-	-	++	++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	II	76
	4.8	-	-	-	-	-	+	++	-	✓	-	-	-	✓	-	-	-	-	✓	-	-	-	III	77
	5.2	-	++	-	-	+++	+++	++	-	-	-	-	-	++	-	-	✓	-	✓	-	-	-	II	78
	4.2	-	-	-	-	-	+	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	I	79
	4.4	-	-	-	-	+	+	-	-	✓	-	-	-	-	-	-	-	-	✓	-	-	-	I	80
	5.6	-	-	+++	+++	-	+++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	III	81
	4.1	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	82
	5.2	-	-	++	-	+++	+++	+++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	I	83
	5.2	-	-	-	-	++	++	-	-	✓	-	-	-	+++	++	-	✓	-	-	-	-	-	III	84
	4.6	-	-	+	-	-	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	I	98
	5.0	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	99
	5.6	-	-	+	-	-	++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	I	100
	4.6	-	-	-	-	-	+++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	I	101
	5.0	-	-	+	-	-	++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	I	102
	6.6	-	-	-	-	-	+++	+++	-	-	-	-	-	++	+	-	-	-	-	-	-	-	III	103
	4.4	-	-	+	-	-	+++	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	I	104
	4.6	-	-	-	+	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	105
	4.8	-	-	+++	++	-	+++	-	-	-	-	-	-	++	-	+++	-	-	-	-	-	-	?	106
G	6.4	++	-	-	-	+++	++	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-	III	8
	5.4	-	-	+++	-	-	-	-	-	++	-	-	-	✓	-	-	-	-	-	-	-	-	III	15
	4.6	-	-	✓	++	+++	+++	-	-	-	-	-	-	✓	-	-	-	-	-	-	-	-	I	33
	5.0	-	-	-	+++	-	-	+++	-	-	-	-	-	++	-	-	✓	-	-	-	-	-	III	39
	5.6	+	-	-	-	+++	+++	-	-	-	-	-	-	-	+++	+++	-	-	-	-	-	-	III	41
	5.4	-	-	-	-	+++	+++	+++	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	III	42
	6.2	-	-	-	-	+++	+++	-	✓	-	-	✓	+	+	-	-	+++	-	-	-	-	-	III	55
	6.4	+	-	-	+++	+++	+++	+++	-	-	-	-	-	++	-	-	-	-	✓	-	-	-	III	57
	4.4	-	-	-	-	+++	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	I	59
	5.4	-	-	-	-	-	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	I	90
	5.6	-	-	+++	+++	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	III	91
	5.6	-	+++	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	II	112
	6.0	-	-	+++	✓	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	115
	5.2	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	118
	6.0	-	-	-	-	-	+++	-	-	+	-	-	-	++	-	-	-	-	-	-	-	-	III	125
	6.2	+++	-	-	++	+++	-	++	-	-	-	-	-	+++	-	-	+++	-	-	-	-	-	III	127
	5.4	-	-	+++	-	++	++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	I	128

TABLE 1.(contd.).

Clinical Group	pH.	Trichomonas	Monilia or other yeasts	HILB	PPLO	Polymorphs	Epithelial cells	Diphtheroids	Anaerobic non-sporing Gram + bacilli	Coliform bacilli	H.influenzae	Anaerobic Gram - bacilli	Staph.aureus	Staph.albus	Non-haemolytic streptococci	Strep.viridans	Strep.faecalis	Strep.pyogenes	Anaerobic streptococci	Cl.welchii	B.subtilis group	N.gonorrhoeae	Flora Type	Serial T.number
	6.4	-	-	+++	✓	+++	+++	-	-	-	-	-	-	+	-	++	-	-	-	-	-	-	III	129
	6.4	-	-	++	✓	+++	++	-	-	-	-	-	-	++	+++	-	-	-	-	-	-	-	III	130
	5.6	-	-	+++	✓	+++	+++	-	-	-	-	-	-	++	-	+++	-	-	-	-	-	-	III	132
	6.6.	-	-	+++	✓	-	+++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	III	140
	5.0	-	-	+++	-	-	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	III	143
	4.6	-	-	++	-	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	162
	6.2	++	-	-	-	+++	++	++	-	-	-	-	-	++	++	-	-	-	+	-	-	-	III	164
	6.8	-	-	-	-	+++	++	-	-	-	-	-	-	++	-	-	+++	-	-	-	-	-	I	173
	6.8	-	-	+++	-	+++	+++	-	-	-	-	-	-	++	-	-	-	-	+++	-	-	-	III	176
	5.8	-	-	+++ ⊙	-	+	+++	-	-	-	-	-	-	+	-	-	-	-	++	-	-	-	III	177
	5.8	-	-	++ ⊙	✓	+++	+++	-	-	-	-	-	-	-	+++	-	+	-	-	-	-	-	III	178
	6.2	+++	-	-	+++	++	++	+++	-	-	-	-	-	+	-	-	-	-	+++	-	-	-	III	185
G	<4.4	-	-	-	-	++	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	I	191
	6.0	+++	-	+++ ⊙	+++	+++	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	III	195
	5.4	-	-	- ⊙	-	++	++	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	I	201
	4.4	-	-	-	-	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	207
	4.8	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	210
	6.8	-	++	+	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	211
	4.6	-	-	+ ⊙	-	++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	226
	5.0	-	-	+++ ⊙	-	+	++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	III	227
	4.8	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	I	228
	6.2	++	-	+ ⊙	-	+++	++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	III	229
	6.6	-	-	-	-	-	+++	+	-	-	-	-	-	+	-	-	-	-	++	-	-	-	III	231
	6.8	-	-	-	-	-	+++	-	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	I	245
	.	-	-	-	-	++	++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	I	259
	5.4	-	-	+++	+++	+	+++	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	III	10
	6.6	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	III	11
GC	6.2	-	-	+++	-	-	+++	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	III	12
	6.6.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	-	-	-	-	-	II	14
	6.8	-	-	-	-	+++	+++	-	-	+++	-	-	-	+++	+++	-	-	-	-	✓	-	-	III	32
	5.0	-	-	+++	-	-	+	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	II	35
	4.4	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	43

TABLE 1.(contd.).

Clinical Group	pH.	Trichomonas	Monilia or other yeasts	HILB	PPLO	Polymorphs	Epithelial cells	Diphtheroids	Anaerobic non-sporing Gram + bacilli	Coliform bacilli	H.influenzae	Anaerobic Gram - bacilli	Staph.aureus	Staph.albus	Non-haemolytic streptococci	Strep.viridans	Strep.faecalis	Strep.pyogenes	Anaerobic streptococci	Cl.welchii	B.subtilis group	N.gonorrhoeae	Flora Type	Serial T. number
	6.6	-	-	-	-	-	++	-	-	++	-	-	-	++	-	-	-	-	-	-	-	-	III	44
	4.4	-	-	-	-	++	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	I	56
	4.6	-	+++	-	-	+++	+++	-	-	✓	-	-	-	-	-	-	✓	-	-	-	✓	-	II	58
	4.6	-	-	-	-	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	61
	4.6	-	-	+++	-	-	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	II	62
	5.6	-	-	+++	-	+++	+++	-	-	-	-	-	-	++	++	-	-	-	-	-	-	-	III	92
	5.2	-	-	-	-	-	++	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-	I	95
	5.6	+	++	+++	✓	-	++	-	-	+	-	-	-	+	+++	-	-	-	-	-	-	-	II	111
	5.0	-	+++	-	✓	-	++	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-	II	113
	4.4	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-	I	116
	5.4	-	-	-	-	+++	+++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	120
	5.1	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	123
	5.2	-	-	+++	-	-	++	-	-	+++	-	-	-	+++	-	-	++	-	-	-	-	-	III	124
	4.8	-	-	-	-	+++	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	I	136
	4.6	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	137
	4.8	-	-	-	-	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	148
GC	5.0	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	152
	5.6	-	-	+++	-	-	++	-	-	-	-	-	-	++	-	++	-	-	-	-	-	-	III	153
	4.4.	-	-	-	-	++	++	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	I	155
	5.0	-	-	-	-	-	+++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	157
	4.8	-	-	-	-	+++	+++	-	-	+	-	-	-	+	-	✓	-	-	-	-	-	-	I	159
	6.8	-	-	+++	-	++	++	-	-	-	-	-	-	-	+++	-	-	-	✓	-	-	-	III	165
	5.4	-	-	-	-	++	++	-	-	-	-	-	-	-	-	-	-	-	+++	-	-	-	I	166
	6.2	-	-	-	✓	+++	++	++	-	-	-	-	-	++	-	-	-	-	++	-	-	-	III	179
	4.4	-	-	-	-	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	184
	4.4	-	-	-	-	++	++	-	-	-	-	-	-	-	✓	-	-	-	-	-	-	-	I	190
	4.4	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	192
	4.8	-	-	-	-	-	++	+	-	-	-	-	-	-	-	-	-	-	++	-	-	-	III	193
	5.8	-	-	-	-	-	++	++	-	-	-	-	-	+	-	-	-	-	-	-	-	-	?	196
	4.4	-	-	-	✓	++	+++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	197
	4.8	-	-	-	-	++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	199
	6.0	-	+++	-	-	++	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	II	203
	4.4	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	208
	4.4	-	-	-	-	+	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	230
	6.6	-	-	-	-	-	++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	I	233

TABLE 1.(contd.).

Clinical Group	pH.	Trichomonas	Monilia or other yeasts	HILB	PPLO	Polymorphs	Epithelial cells	Diphtheroids	Anaerobic non-sporing Gram + bacilli	Coliform bacilli	H.influenzae	Anaerobic Gram - bacilli	Staph.aureus	Staph.albus	Non-haemolytic streptococci	Strep.viridans	Strep.faecalis	Strep.pyogenes	Anaerobic streptococci	Cl.welchii	B.subtilis group	N.gonorrhoeae	Flora Type	Serial T. number
PM	6.8	-	-	-	+++	-	+	++	-	✓	-	-	-	-	-	-	✓	-	-	-	-	-	II	31
	6.6	-	-	++	-	++	++	-	-	-	-	-	-	++	-	+	-	-	-	-	-	-	III	94
	6.0	-	-	+++	-	-	+++	+++	-	-	-	-	-	+	+	-	-	-	-	-	-	-	III	119
	5.1	-	-	- ⊙	-	+++	+++	-	-	-	-	-	-	++	-	++	-	-	-	-	-	-	III	121
	6.4	++	-	-	+++	+++	++	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	III	122
	6.6	-	-	+	++	++	+++	-	-	+	-	-	-	++	-	+	-	-	-	-	-	-	III	126
	6.0	-	-	+++	✓	+++	+++	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	III	131
	5.8	-	-	-	-	+++	++	+++	-	+	-	-	-	++	-	-	-	-	+++	-	-	-	111	135
	6.0	-	-	+++	-	-	+	+++	-	-	-	-	-	++	-	-	-	-	-	-	++	-	III	139
	6.6	-	-	-	-	++	++	+	-	-	-	-	-	++	-	-	-	-	+	-	-	-	III	151
	6.4	-	-	-	++	-	+++	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	III	156
	6.8	-	-	++	-	+++	+++	+	-	-	-	-	-	+	✓	-	-	-	-	-	+	-	III	163
	6.6	-	-	+++	-	++	++	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	III	174
	6.6	-	-	-	-	+++	+++	+++	-	-	-	✓	-	-	-	-	-	-	++	-	-	-	III	180
	6.8	-	-	-	+++	+++	++	++	-	-	-	-	-	-	-	-	-	-	+++	-	-	-	III	202
	6.6	-	-	-	-	-	++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	I	209
	5.8	++	-	-	-	+++	-	-	-	-	-	-	-	+	-	-	+++	-	-	-	-	-	III	223
	6.2	-	-	-	-	+++	++	+++	-	-	-	-	-	+++	-	-	-	-	+++	-	-	-	III	232
PMC	6.8	-	-	-	-	-	+	-	-	✓	-	-	-	+	-	-	✓	-	-	-	-	-	III	13
	6.8	-	-	-	-	-	+	-	-	✓	-	-	-	-	-	-	+++	-	-	-	-	-	III	37
	6.4	-	-	+++	-	+++	+++	-	-	+	-	-	-	++	-	-	+++	-	-	-	-	-	III	60
	6.8	-	-	-	-	-	++	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	I	93
	4.4	-	-	-	-	-	+++	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	I	117
	6.4	-	-	+++	-	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	III	147
	6.8	-	-	-	-	+	++	++	-	+	-	-	-	+	-	-	-	-	+++	-	-	-	III	150
	6.6	-	-	-	+++	-	+++	-	+++	-	-	-	-	+	-	-	-	-	-	-	-	-	III	158
	6.8	-	-	-	-	-	+	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	?	160
	6.8	-	-	-	-	-	++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	?	180
	5.0	-	-	-	-	++	+++	+++	-	-	-	-	-	++	-	-	+	-	-	-	-	-	?	187
	6.6	-	-	-	-	+++	+++	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	III	188
	6.2	-	-	+	-	-	++	-	-	++	-	-	+++	++	++	-	-	-	-	-	-	-	?	200
	5.0	-	+	+++	-	+++	++	+	-	-	-	-	-	-	-	-	-	-	++	-	-	-	III	204

TABLE 1.(contd.).

Clinica Group	pH.	Trichomonas	Monilia or other yeasts	HILB	PPLO	Polymorphs	Epithelial cells	Diphtheroids	Anaerobic non-sporing Gram + bacilli	Coliform bacilli	H.influenzae	Anaerobic Gram - bacilli	Staph.aureus	Staph.albus	non-haemolytic streptococci	Strep.viridans	Strep.faecalis	Strep.pyogenes	Anaerobic streptococci	Cl.welchii	B.subtilis group	N.gonorrhoeae	Flora type	Serial T. number
PN	4.4	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	161
	6.4	-	-	-	-	+	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	206
	6.4	+	-	-⊙	-	+++	++	-	-	+	-	-	-	+	-	-	-	-	++	-	-	-	III	225
	6.8	-	-	-	-	++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	240
	6.6	-	-	-	-	-	+++	+	-	-	-	-	-	-	++	-	-	-	-	-	-	-	III	249
	6.6	-	-	+++	-	+++	++	-	-	+++	-	-	-	-	-	-	+++	-	-	-	-	-	III	250
	6.4	-	-	-	-	++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	?	254
PD	6.2	-	-	+++	-	+	++	+++	-	-	-	-	-	-	++	-	-	-	-	-	-	-	III	235
LD	6.8	-	-	++	++	-	+++	+	-	-	-	-	-	++	-	-	-	-	-	-	-	-	?	215
PPU	.	-	-	+++	✓	+++	+++	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	III	181
	.	-	-	-	-	+++	++	-	-	-	+++	-	-	+	-	-	-	-	-	-	-	-	?	280
	.	-	-	-	-	++	-	-	-	-	+++	-	-	+	-	-	-	-	-	-	-	-	III	281
UC	6.8	-	-	-	-	++	++	+++	-	++	-	-	-	++	-	-	-	-	-	-	-	-	III	114
	4.4	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	222

KEY TO TABLE 1.

PP = Puerperal pyrexia

PC = Puerperal control

AN = Ante-natal

G = Gynaecological

GC = Gynaecological control

PM = Post-menopausal

PMC = Post-menopausal control

PN = Post-natal

PD = Puerperal discharge

LD = Discharge during labour

PPU = Pre-pubertal

UC = Clinical group uncertain

? = Flora type uncertain

⊙ = Non-haemolytic strain present, otherwise resembling HILB

✓ = Organism isolated from fluid culture

\+ = scanty

++ = moderate number

+++ = numerous

T141 and T142 were inadvertently missed, and do not represent any specimen.

The total numbers of isolations of different organisms in culture, or detection in films (excluding Döderlein's bacilli, which can be assumed to be present in flora types I and II) are given below, in descending order of frequency.

Name of organism.	No. of times isolated
Staph.albus	157 (63.6%)
HILB	95 (38.1%)
Diphtheroids	64 (25.9%)
PPLO	45 (18.4%)
Coliform bacilli	40 (16.2%)
Anaerobic strep.etc.	35 (14.2%)
Non-haemolytic strep.	32 (12.9%)
Strep.faecalis	32 (12.9%)
Monilia or yeasts	18 (7.3%)
Strep.viridans	15 (6.1%)
Trichomonas	13 (5.3%)
Anaerobic Gram -ve.bacilli.	8 (3.2%)
Staph.aureus	7 (2.8%)
Bacilli of subtilis group.	4 (1.6%)
H.influenzae	3 (1.2%)
Cl.welchii	2 (0.8%)

Total number of specimens examined = 247.

No Strep.pyogenes or N.gonorrhoeae were isolated during the investigation.

It will be seen that, apart from Staph.albus, HILB is the commonest organism, followed by diphtheroid bacilli, PPLO and other organisms. True H.influenzae is confirmed to be rare in high vaginal specimens, only one isolation occurring in a woman of reproductive age, namely T277, a case of PP. The other 2 were in young girls.

Comparative Incidence of HILB in the various clinical groups.

The incidence of HILB in the various groups is shown in Table 2 and Fig. 3. The highest incidence was recorded in the puerperal pyrexia group at 71%. Next came the gynaecological group, 43%, followed by the post-menopausal group, 39% and the 3 control groups, 31 - 21%.

The X^2 test was carried out and the probability of the observed differences in incidence being due to chance was shown to be negligible ($X^2 = 29.23$, $P = <0.01$). For the statistical calculations themselves, see Appendix, Tables 79 and 80.

When this test was applied to the control groups only, however, (ante-natal, puerperal control, gynaecological control and post-menopausal control) X^2 was found to be only 0.84 ($P = >0.8$) i.e. there was no significant difference in incidence of HILB in the various control groups.

Association of HILB with puerperal pyrexia.

It will be seen that a very much higher incidence of HILB occurred in the PP group (71%) than in the PC group (30.8%). The Standard Error of the difference between these 2 proportions (see Appendix) was found to be ± 12.2%. The observed difference was 40.2%, i.e. 3¼ times S.E. Taking the conventional level of 2 x S.E. as the minimum for significance, the observed difference is highly

TABLE 2.

Incidence of H.influenzae-like bacilli in the Various Groups.

	No. of cases in Group	No. HILB+ve	% HILB+ve
Puerperal Pyrexia Group	45	32	71.0%
Puerperal Control Group	26	8	30.8%
Ante-natal Group	44	12	27.3%
Gynaecological Group	42	18	42.8%
Gynaecological Control	42	9	21.4%
Post-menopausal Group	18	7	38.9%
Post-menopausal Control Group.	14	4	28.6%
TOTAL	231	90	38.9%

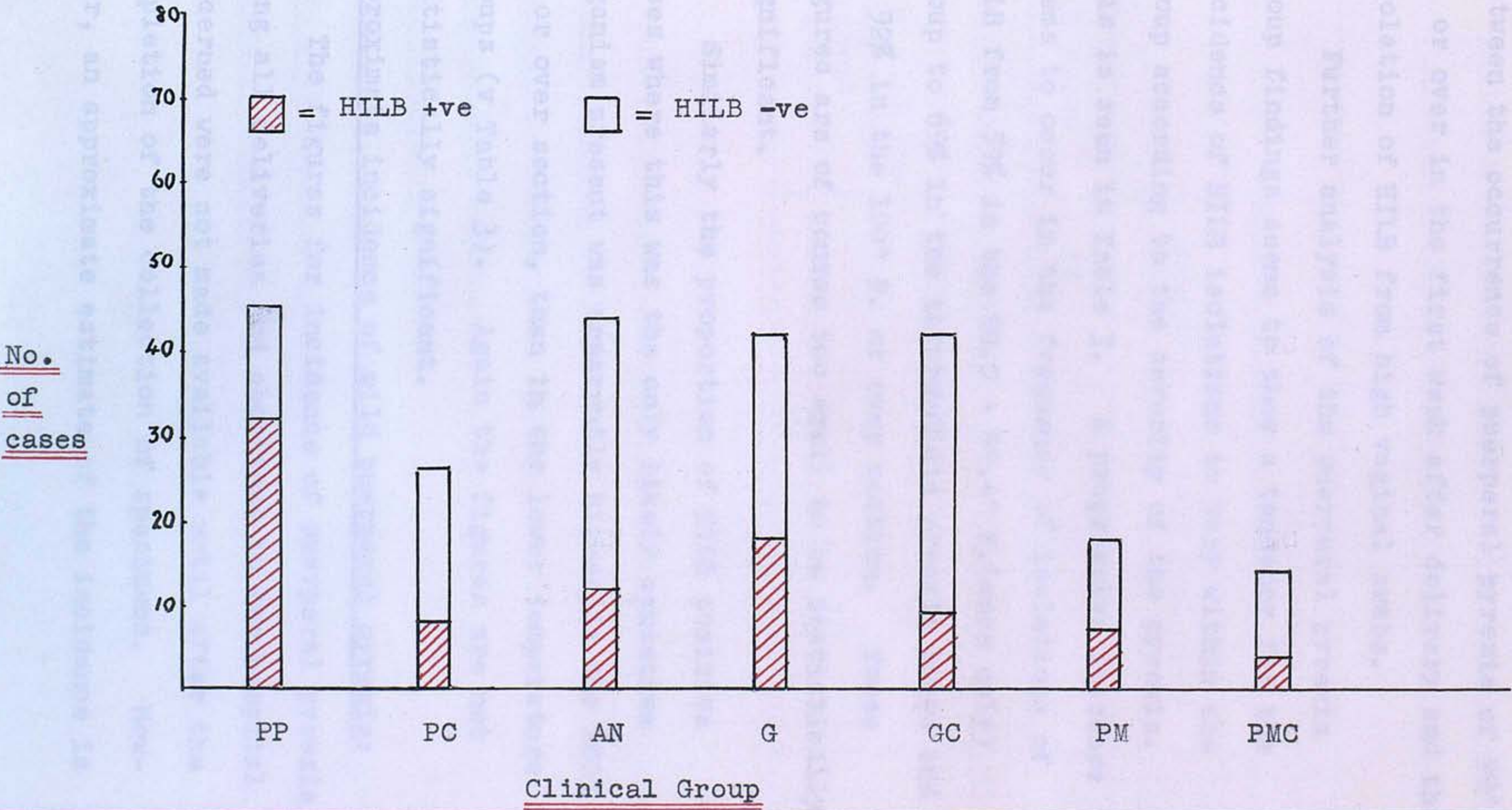

Fig. 3.

significant. An association is therefore shown between the occurrence of puerperal pyrexia of 99° F. or over in the first week after delivery and the isolation of HILB from high vaginal swabs.

Further analysis of the puerperal pyrexia group findings seems to show a tendency for the incidence of HILB isolations to vary within the group according to the severity of the pyrexia. This is seen in Table 3. A progressive increase seems to occur in the frequency of isolations of HILB from 59% in the 99.0 - 99.4° F.(once only) group to 69% in the intermediate pyrexia range and to 92% in the 100° F. or over section. These figures are of course too small to be statistically significant.

Similarly the proportion of HILB positive cases where this was the only likely causative organism present was apparently higher in the 100° F. or over section, than in the lower temperature groups (v Table 3). Again the figures are not statistically significant.

Approximate incidence of mild puerperal pyrexia:

The figures for incidence of puerperal pyrexia among all deliveries and abortions in the hospital concerned were not made available until after the completion of the collection of specimens. However, an approximate estimate of the incidence is

TABLE 3.

Incidence of HILB and other recognised pathogens* according to the degree of Puerperal Pyrexia.

	Temperature		
	99 - 99.4°F. once only	99 - 99.4°F. more than once or 99.5 - 99.9°F.	100°F. or more.
No. of cases	17	16	12
No. HILB +ve.	10 (59%)	11 (69%)	11 (92%)
No. of HILB +ve. showing other recognised pathogens. *	2	1	0

* Other recognised pathogens are defined as follows:

Strep.pyogenes isolated.

Anaerobic streptococci isolated.

Cl. welchii isolated.

H. influenzae isolated.

Staph. pyogenes isolated in moderate or heavy growth.

E. coli isolated in heavy growth.

Strep. faecalis isolated in heavy growth.

obtained from the figures for a later month, acting on the assumption that all cases had a high vaginal swab sent to this laboratory.

Total number of deliveries and abortions in
January 1959 = 151.

Total number of puerperal pyrexias, as determined
by number of specimens received by the
laboratory = 57.

∴ Incidence of pyrexia = 38%.

The number 57 was exclusive of those whose accompanying urine specimens showed an obvious urinary infection with pus. The source of pyrexia may have been extra-genital, although uterine infection was suspected at the time of sending the specimen.

Proportion of cases notifiable:

The definition of notifiable pyrexia current at the time (January 1959) was "a temperature of 100.4° F. once within 14 days of childbirth or miscarriage". The number attributable to the genital tract was 7, i.e. about 1/8 of the total pyrexias as determined above.

Association of HILB with leukorrhoea.

From Table 2 it can be seen that the incidence of HILB in the gynaecological group (42.8%) is exactly double that in the control group (21.4%). Statistical analysis shows that the S.E. of the Difference between these proportions is ± 10.2%,

while the observed difference is 21.4%, i.e., just over twice the S.E. The difference can therefore be said to be statistically significant but not striking. When the incidence of HILB in the groups G + PM (25 out of 60 = 42%) is compared with that in the groups GC + PMC (13 out of 56 = 23%), the observed difference is 19%, the S.E. of the difference being ± 8.7%, so that the observed difference is more than 2 x S.E. i.e., this difference is also statistically significant. The difference in incidence between PM and PMC groups is obviously not significant, however.

Relation of HILB to trichomonas, yeast and other infection.

Table 4 shows the relation, for the combined groups, of HILB to trichomonas, yeasts and other recognised pathogens. The incidence of HILB in trichomonas positive cases is seen to be about 20% and in cases with other recognised pathogens (not yeasts) about 25%. These compare with the total HILB incidence of 38.9% for the 7 main groups, the difference not being significant in either case. On the other hand, comparatively few instances were found of isolation of HILB from yeast infections (2 out of 18). This proportion of 11% is significantly lower than the proportion of 40.6% for yeast-negative specimens. The observed difference

TABLE 4.

Relation of HILB isolation to the presence of other pathogens.

Pathogen present	No. of cases	No. HILB +ve.	% +ve.
Trichomonas vaginalis	14	3	21%
Yeasts	18	2	11%
Other recognised pathogens*	41	10	24%

* Defined as in table 3.

is 29.6% and the S.E. of difference is ± 11.9%, i.e. observed difference is 2½ x S.E.

Table 5 shows the incidence of HILB isolations in 4 different pH groups of vaginal secretions. The majority occurred in groups pH 5.1 - 7.0. It is surprising how many were isolated from secretions with quite low pH levels (21.6% of 74 in the pH 4.0 - 5.0 group). As would be expected from the type of flora associated with this group, a higher proportion of these specimens gave a scanty (+) growth (about 1/3) than the pH 5->7 group (about 1/8). Table 5 shows that PPLO isolations also occur more frequently from secretions in the upper pH range (6.1 - 7.0) than in the lower ranges.

Table 6 shows that the great majority of all isolations of HILB occur in +++ growth. This applies equally in disease and control groups (although this fact is not shown in the table). The incidence of HILB isolations was highest (53.2%) from specimens whose microscopic appearance was that of type III (so-called "mixed bacterial") flora (v. Cruickshank and Baird, (1929 - 1930), for definition of flora types). There was, however, a surprisingly large number isolated from type I flora (15.4%). Most of these were + or ++ growth, and in only 2 cases was a heavy +++ growth obtained from this flora type (both of these, and the majority of other isolations from type I flora,

TABLE 5.

Relation of HILB and PPLO isolations to pH of vaginal secretion.

	pH group			
	4.0 - 5.0	5.1 - 6.0	6.1 - 7.0	7.0
No. of cases	74	46	100	8
No. HILB +ve.	16 (21.6%)	22 (48%)	47 (47%)	2 (25%)
No. PPLO +ve.	7 (9.4%)	9 (19.6%)	25 (25%)	0
No. HILB +ve. (+ growth only)	5	1	7	0

\+ growth = scanty growth, with few widely separated colonies in well only.

++ growth = moderate growth, with numerous closely set but separate colonies in well, but few or no colonies in the periphery.

+++ growth = heavy growth confluent in well, and numerous colonies after sucessive stroking, on remainder of plate.

TABLE 6.

Incidence and amount of growth of HILB, and incidence of PPLO, according to Flora Types I, II and III.

	Amount of Growth	Flora Type			Total
		I	II	III	
No. of cases		78	17	115	210
No. HILB +ve.	+	7	1	3	11(5.2%)
	++	3	0	7	10(4.8%)
	+++	2	4	57	63(30.0%)
	Total	12(15.4%)	5(29.4%)	67(53.2%)	84(40.0%)
No. PPLO + ve.		4(5.1%)	3(17.7%)	35(30.5%)	42(20.0%)

+ , ++ , +++ growth:- see key to Table 5.

occurred in pregnant women).

A similar tendency can be seen for PPLO isolations, 30.5% of type III flora specimens yielding this organism as against 17.7% of type II, and 5.1% of type 1.

Table 7 shows the relation between isolations of HILB and PPLO from all specimens in the main groups. The incidence of PPLO isolations among HILB positive specimens (27.8%) was more than twice as high as among HILB negative specimens (11.4%). The observed difference between these percentages (16.4%) is highly significant, being three times the S.E. of the difference (± 5.15).

There is therefore a definite association between the presence of HILB and PPLO in the specimens as a whole. When the PPLO incidences are broken down into each clinical group, the same association is apparent, being most obvious in the puerperal control and gynaecological groups (v. Table 8). However, in the puerperal pyrexia group, the incidence was almost the same in HILB positive and negative specimens.

This association could be due to a common association between the 2 organisms on the one hand and the vaginal pH and flora type on the other, as it has already been pointed out that both HILB and PPLO are associated with the higher pH ranges and

TABLE 7.

Relation of HILB to PPLO in all main groups and in 2 pH groups.

	No. of cases	No. HILB+ve	No. HILB-ve	No. PPLO+ve in HILB+ve group	No. PPLO+ve in HILB-ve group
All cases	231	90	141	25(27.8%)	16(11.4%)
pH of specimen* 5.4 or less	93	22	71	4(18.2%)	3(4.2%)
pH >5.4	134	64	70	21(32.8%)	11(15.7%)

* pH readings were not obtained from all specimens.

TABLE 8.

PPLO incidence in HILB+ve and -ve cases in the various groups.

Clinical group	No. of HILB+ve	HILB +ve		No. of HILB-ve	HILB-ve	
		No. PPLO	%		No. PPLO	%
PP	32	8	25%	13	3	23%
PC	8	2	25%	18	0	0%
AN	12	2	17%	33	2	6%
G	18	7	39%	24	4	17%
GC	9	2	21%	33	3	9%
PM	7	3	43%	11	4	36%
PMC	4	0		10	1	10%

with type III flora rather than type I or II. However, the relationship still seems to hold good when the pH range is split into 2 separate parts, pH 5.4 or under, and over 5.4 (v.Table 7). The percentage of PPLO positives among HILB positive specimens of pH 5.4 or less is 18.2%, and among HILB negative specimens only 4.2%. In the higher pH range the corresponding percentages are 32.8% and 15.7%, the 2 proportions even in this case being significantly different (observed difference 17.1%, S.E. $\pm$ 7.45). There seems little doubt therefore that these 2 organisms have a real association between them in high vaginal specimens.

Relative incidence of HILB in vaginal and cervical lesions.

There were 36 cases where the presence of vaginitis or cervical erosion or cervicitis was definitely recorded. These are shown in Table 9.

Table 9.

	No. of cases	
	Vaginitis	Cervical erosion or cervicitis
HILB +ve	5 (31%)	9 (37.4%)
Total	16	24

There is a higher incidence of HILB in cervical cases than in those with vaginitis only, (37.4% as against 31%) but the figures are too small for statistical significance. Of the 5 vaginitis cases from which HILB were isolated 3 yielded other recognised pathogens, but of the corresponding 9 cervicitis cases only 2 gave other recognised pathogens, 7 having HILB as the only likely causative organism.

C H A P T E R III

MORPHOLOGICAL AND CULTURAL CHARACTERS

CHAPTER III. MORPHOLOGICAL AND CULTURAL CHARACTERS.

Section 1. Microscopic morphology.

Methods:

For examination of the strains for shape, size, arrangement, Gram reaction and metachromatic granules, a small portion of a blood agar plate was heavily inoculated with a 48 hours 37° C. culture grown on the same type of medium. Duplicate plates were set up and incubated at 37° C. After 24 hours, thin films were made, fixed by passing 3 times through a bunsen flame, and stained Gram. For decolourisation, alcohol was used as described in Chapter II. By this means, any tendency to retention of the methyl violet by the organism could be detected. It was found that the reaction to Gram as seen in routine stained films from high vaginal specimens, was rather unreliable, presumably owing to the high protein content of the secretion. Organisms which appeared as mixtures of Gram negative and Gram positive bacilli in the original film, when cultured, filmed and stained showed up as entirely Gram negative bacilli. On the other hand, use of acetone would have carried too much risk of over-decolourisation, with resultant inclusion in the series of organisms which were really diphtheroid bacilli. Slides were examined using the oil-immersion lens, final magnification being

x 1000.

The filming and staining of the cultures was repeated after a further 48 hours incubation and the microscopic characters of the organism were recorded from both 24 and 72 hours cultures. The results shown in Table 10 are, unless otherwise specified, obtained from 72 hours cultures. Usually the appearance of the bacilli remained unchanged during this period of incubation, but where change occurred, this is shown in the table.

Metachromatic Granules:

These were tested for by staining films made from 72-hour 37° C. aerobic blood agar cultures, inoculated as described above. They were not so marked nor so well-defined as those of the diphtheroid group.

The effect on granule production of sugar-containing medium was investigated as follows:-

10% filtered serum digest agar plates were prepared (v. Appendix No. 46) with glucose, maltose, arabinose, xylose, laevulose, galactose, dextrin or starch incorporated in 1% concentration. The plates were heavily inoculated with a 48-hour 37° C. aerobic culture on blood agar, and were incubated for 4 days aerobically at 37° C. Films were made of the resulting growth (if any) and stained Gram. The presence of granulation was

noted from the Gram positive appearance of the swollen terminal portions of some of the bacilli.

Further experiments were done using blood agar to which 0.25% maltose, glucose or mannite had been added. Inoculation was as before, but incubation was for 3 days and Albert's stain was used to demonstrate granules. One strain was grown for 48 hours in 1% maltose plasma digest broth (Appendix No.37) and films of the growth made and stained Gram. This film was photographed (v. under results).

Spores:

The same films were used as were made for the study of shape, size, Gram reaction etc., and the absence of spores noted in each case, using x 1000 magnification.

Capsules:

As capsules usually grow better in a medium containing carbohydrate, 1% maltose was incorporated in the blood agar and thick plates poured. The plates were inoculated as described above and incubated for 5 days at 37° C. Indian ink slide preparations were made with the culture and examined for capsules using the oil-immersion lens.

Motility:

Blood agar broth tubes were made as described in the Appendix (No. 20), consisting of plasma

digest broth superimposed on blood agar. A heavy inoculum from a 48-hour culture on blood agar was implanted in the surface of the blood agar, and emulsified in the supernatant fluid. After 24 hours aerobic incubation the tubes were shaken, a loopful of the broth placed on a slide and a cover-glass added. This was sealed with melted vaseline and the preparation examined by oil-immersion lens (final magnification x 1000). This was necessary in view of the small size of the bacillus. The diaphragm was stopped down. A few strains were tested after growing 24 hours in 0.1% sloppy agar superimposed on blood agar (Appendix No. 21).

Results:

General Description: (v. Figs. 8 and 9).

HILB is a small Gram negative non-sporing bacillus resembling H.influenzae when in its cocco-bacillary form. Its size varies from 0.5 x 0.3μ to 2 - 3 x 0.5μ, the average being 1.0 - 1.5μ x 0.4μ. Like H.influenzae, it is pleomorphic in many cases, with both small cocco-bacillary and larger bacillary forms seen. It does not, however, show the long filaments characteristic of the rough form of H.influenzae. It has a tendency to grouping in parallel rows and to show some angling with two bacilli meeting at an acute angle at their ends, in a manner resembling that of diphtheroid

bacilli. Some strains produce rather ill-defined granules, resembling the volutin granules of diphtheroid bacilli, and there is associated with this a tendency to retain Gram's stain in occasional individual bacilli. These tendencies are much increased by growth in fluid medium or in media containing a fermentable carbohydrate.(Figs.10,11). Some strains have a degenerated appearance when stained by Gram's method, with ghost-like bulbous forms reminiscent of L-forms.

When seen in vaginal smears, it is often in very large numbers and is in association both with epithelial cells and polymorphs. Some epithelial cells become coated with numerous cocco-bacilli and begin to disintegrate, when they show the appearance of the so-called "clue-cell" of Gardner and Dukes (1955) (v. Figs. 4,5). Occasionally it is seen in large numbers packed into the cytoplasm of a polymorph (v.Figs. 6,7) but more often only the remains of a cell are seen with masses of HILB in it. Presumably disintegration of the polymorphs follows rapidly after the appearance seen in Fig. 6 . In vaginal smears HILB often has a partially Gram positive appearance, so that there appear to be 2 different organisms present, one Gram positive, the other Gram negative. These are thought to be the same organism, the Gram positive appearance

being the result of a combination of the known tendency of HILB to retain Gram under certain conditions, and the presence of much albuminous material in the secretion, preventing even decolourisation. When HILB are emulsified in artifical plasma mixtures and filmed, this Gram positive tendency is increased, due to prevention of adequate decolourisation by the coagulated plasma proteins.

Details of morphological features are given in Table 10. From this table, the following morphological groups can be obtained:-

Groups on the basis of morphology.

1. Small, non-diphtheroid, and cocco-bacillary. Completely Gram negative.

T numbers, 20, 30, (72 hours incubation), 34 (72 hours), 86, 87, 102, 121, 124, 149. Total 9.

2. Medium-sized, somewhat diphtheroid arrangement, completely Gram negative.

T.numbers 7, 12, 15, 30,(24 hours), 33, 34 (24 hours), 35, 46, 62, 66, 73, 88, 92, 94, 104, 111, 126, 131, 132, 143, 145, 162, 168. Total 23.

3. Small, somewhat diphtheroid arrangement, completely Gram negative.

T numbers 140, 144, 147, 169. Total 4.

4. Showing marked pleomorphism.

T numbers 100, 109, 115, 149 (24 hours). Total 4.

5. Degenerated appearance.

T numbers, 7, 121, 124. Total 3.

TABLE 10.

Morphological details of HILB.

	Size			Shape			Arrangement.		Gram		Degenerated	Met. Gran.
	S	M	L	Pl	Sl	C-B	D	ND	-ve	partly +ve		
T7	-	+	-	-	-	-	+	-	+	-	+	-
T12	-	+	-	-	-	-	+	-	+	-	-	-
T15	+	+	-	-	+	-	+	-	+	-	-	-
T20	+	-	-	-	+	-	-	+	+	-	-	-
T30 (24hrs.)	-	+	-	-	-	-	+	-	+	-	-	-
T30 (72hrs.)	+	-	-	-	-	-	-	+	+	-	-	-
T33	-	+	-	-	-	+	+	-	+	-	-	-
T34 (24hrs.)	-	+	+	-	+	-	+	-	+	-	-	-
T34 (72hrs.)	+	-	-	-	-	-	-	+	+	-	-	-
T35	-	+	-	-	-	-	+	-	+	-	-	+
T38	-	+	-	-	-	-	+	-	-	+	-	+
T46	+	-	+	-	+	-	+	-	+	-	-	-
T62	-	+	-	-	-	-	+	-	+	-	-	-
T66	+	+	-	-	+	-	+	-	+	-	-	-
T73	-	+	-	-	-	-	+	-	+	-	-	-
T83	+	+	-	-	+	-	+	-	-	+	-	-
T86	+	-	-	-	+	-	-	+	+	-	-	+
T87	+	-	-	-	-	+	-	+	+	-	-	-
T88	+	+	-	-	-	-	+	-	+	-	-	+
T91	+	+	-	-	-	-	-	+	+	-	-	+
T92	-	+	-	-	+	-	+	-	+	-	-	-
T94	+	+	-	-	+	-	+	-	+	-	-	-
T98	+	+	-	-	-	-	-	+	+	-	-	-
T100	-	-	-	+	-	-	+	-	+	-	-	-
T102	+	-	-	-	-	-	-	+	+	-	-	-
T104	-	+	-	-	+	-	+	-	+	-	-	+
T109	-	-	-	+	+	-	-	+	+	-	-	-
T111	+	+	-	-	-	-	+	-	+	-	-	-
T115	-	+	+	+	-	-	-	+	+	-	-	-

TABLE 10.(contd.).

	Size			Shape			Arrange-ment		Gram		Degen-erated.	Met. Gran.
	S	M	L	Pl	Sl	C-B	D	ND	-ve	partly +ve		
T121	+	-	-	-	-	-	-	+	+	-	+	-
T124	+	-	-	-	-	-	-	+	+	-	+	±
T126	+	+	-	-	+	-	+	-	-	+	-	-
T131	+	+	-	-	-	-	+	-	+	-	-	+
T132	+	+	-	-	+	-	+	-	+	-	-	-
T140	+	-	-	-	+	-	+	-	+	-	-	-
T143	-	+	-	-	+	-	+	-	+	+	-	±
T144	+	-	-	-	+	-	+	-	-	+	-	-
T145	-	+	-	-	+	-	+	-	+	-	-	-
T147	+	-	-	-	+	-	+	-	+	-	-	-
T149 (24hrs.)	+	-	-	+	-	-	-	+	-	+	-	+
T149 (72hrs.)	+	-	-	-	+	-	-	+	+	-	-	-
T162	-	+	-	-	+	-	+	-	+	-	-	±
T168	+	+	-	-	+	-	+	-	+	-	-	±
T169	+	-	-	-	-	-	+	-	+	-	-	+

S = small.
M = medium-sized.
L = large.
Pl = pleomorphic.
Sl = slender.
C-B = cocco-bacillary.
D. = diphtheroid.
ND = not diphtheroid.
Met. Gran = metachromatic granules.

6. Metachromatic granules demonstrated.

T numbers 35, 38, 86, 88, 91, 104, 124, 131, 143, 149, (24 hours), 162, 168, 169. Total 13.

7. Showing Gram positive tendency:

T numbers 38, 83, 126, 143, 144, 149 (24 hours). Total 5.

These groups are of doubtful significance, as it was found by later observations that variations occurred in the morphological features of indivi- strains according to the conditions. Thus, most strains, when grown anaerobically on solid medium, showed the small cocco-bacillary shape, with no apparent diphtheroid tendency, even though this was shown by the same strain in aerobic cultures. Also, certain strains became more Gram positive through time, e.g. T 34 which after repeated sub-cultures, while retaining the characteristic colonial morphology and cultural characters of HILB, became partially Gram positive even on blood agar.

Effect of fermentation on granule-production:

Table 11.

Strain	Maltose		Arabinose		Xylose		Galactose	
	F.	G.	F.	G.	F.	G.	F.	G.
T 220	+	+	+	+	-	-		
T 262			+	+			-	-

F = fermentation.

G = granules.

Table 11 shows the effect of fermentation on granule production by 2 strains using serum sugar agar. A blank indicates that no growth occurred, - that growth occurred but that either fermentation or granulation had not. Granule-production occurred when the sugar was fermented, but failed to occur when growth was unaccompanied by fermentation.

Table 12 shows the effect of fermentation on granulation with 5 further strains, this time grown on blood agar containing maltose, glucose or mannite, in 0.25% concentration. (v. Fig. 13 also).

Table 12.

Strain	Sugar, in blood agar					
	Maltose		Glucose		Mannite	
	F.	G.	F.	G.	F.	G.
693	+	-	+	+	-	-
623	+	+	+	+	-	-
880	+	-	+	+	-	-
239	+	-	+	+	-	-
837	+	+	+	+	-	-

F = Fermentation.

G = Granulation.

All the cultures grew well, but granules could be seen only when the sugar in the medium was fermented. Glucose appeared to be particularly

effective in causing granulation.

The effect of fermentation in a fluid medium is seen in Fig.12 . Strain T238 was grown for 48 hours in 1% maltose plasma broth. Granulation is seen in the photograph.

Spores:

After 24 hours incubation, T numbers 7, 12, 15, 30, 33, 34, 35, 38, 46, 62, 66, 83 and 86 were examined for spores and found to be all negative. After 72 hours incubation, all the strains shown in Table 10 were examined and found negative.

Motility:

All the strains in Table 10 except T144 and T145 were tested for motility by the technique described above and no evidence of motility found. Two strains tested in sloppy agar/blood agar were also negative after 24 hours' incubation.

Capsules:

All strains in Table 10 except T numbers 126, 131, 132, 140, 143, 144, 145 and 149 were tested by the method described and found to lack capsules in every case.

Section 2: Colonial morphology:

A. After 24 hours incubation at 37° C.

i. Blood agar culture, aerobic.

Individual colonies are usually barely visible even with a hand lens. If a heavy inoculum has been used for the well and if conditions are optimum,

growth may be observed easily ih the well with the naked eye. Under conditions of primary isolation, however, it is quite common for no visible growth to be seen after 24 hours, even in the well. Faint haemolysis may be visible in the well, but this is usually masked by the thickness of the blood agar.

ii. Blood agar culture, aerobic plus 10% CO_2

and

iii. Blood agar culture, anaerobic incubation.

Both are substantially the same as i, except that in certain cases, primary isolatioh is successful only on anaerobic culture or only on aerobic. In all cases, however, second and subsequent cultures grow with similar ease (or lack of ease) by all 3 methods. In a few cases, a definitely improved volume of culture is obtained by the use of 10% CO_2.

iv. 10% serum digest agar culture, aerobic.

The faintest haze may be seen in the well, and the 1st and 2nd strokes may be visible also with a hand lens. This medium is very variable, according to the batch of serum, and there may be no growth visible.

v. Autoclaved blood agar, aerobic.

Very similar to blood agar except for lack of haemolysis.

vi. Boiled blood agar, aerobic.

Same as v., but not so reliable, occasional batches failing to support growth.

vii. 1% maltose blood agar, aerobic.

Growth slightly better than i., if successful.

B. After 48 hours incubation at 37°C.

i. Blood agar, aerobic. (v. Fig. 23).

Under optimum conditions, individual pin-point colonies can now be clearly seen with the naked eye, together with a small zone of haemolysis, partially masked by the thick layer of blood agar. If a thin plate has been used, and growth is successful, the haemolysis appears much more marked. It is now possible to pick off single colonies for isolation of pure cultures, preferably with the aid of a hand lens and transmitted light. Better results are, however, obtained by waiting until 3 days have elapsed.

The character of the individual colony can now be seen with a plate microscope or the low power of an ordinary microscope. Its size is approximately 0.1 - 0.2 mm. in diameter, it is smooth, rounded with entire edge. It is transparent or nearly so. Its consistency is soft and creamy, although this is difficult to tell with such a small

colony, and the feel of the culture can be got better from the well. When the growth is scraped together it becomes greyish white and it apparently emulsifies quite easily in water or saline. However, close inspection, with a hand lens, reveals that the emulsion is in reality rough and no amount of shaking will make it smooth, short of partial disruption with grade 9 ballotini.

The colony, when scraped away, leaves a minute hollow, revealed only by close inspection with a hand lens, with oblique reflected light.

The diameter of the zone of haemolysis is approximately 3 x that of the colony. Its edge is moderately well defined and, if the plate is a thin one, microscopic examination (low power) shows that haemolysis is complete.

Occasional non-haemolytic strains, e.g. T35NH, T217NH, were encountered, which were identical to the haemolytic ones in every other respect as far as could be ascertained, though no information is available as to their share of pathogenic properties.

ii. Blood agar, + CO_2 and iii. anaerobic.

Similar to i, except that occasionally growth was slightly better and haemolysis more pronounced.

iv. Serum agar, v. autoclaved blood agar,

vi. boiled blood agar.

Essentially the same colony characters, although these did not match blood agar for consistent per-

formance. Visibility of the colonies was poor compared with those on blood agar due to lack of haemolysis as a guide.

vii. <u>1% maltose blood agar</u>.(Figs. 19 and 20).

Colony bigger and more opaque, when growth was successful. Haemolytic zone slightly wider. Scraped up growth has the same consistency and colour as on blood agar, but is more abundant.

C. <u>After 72 hours incubation at 37° C</u>. (Figs.14-17, 21,22,24 & 25).

Characters remain the same throughout except for:-

(1) Larger size; colony on blood agar is now 0.3mm. in diameter and on 1% maltose blood agar 0.5 - 0.8 mm. These sizes are slightly larger if steps against atmospheric drying have been taken, e.g., by incubation in a sealed jar.

(2) Colonies become more opaque and greyish-white in colour, but retain their shape. The slight pitting of the medium beneath a colony becomes more pronounced.

(3) Acid production on the maltose blood agar may lead to a brownish discolouration of the medium, especially where growth is confluent. A loopful of bromthymol blue indicator will turn yellow when placed there.

D. <u>After 4 or more days incubation</u>.

The colonies do not get any bigger except in

sugar media such as maltose blood agar, if fermentation is occurring. The medium becomes brown and colonies, when scraped off, are discoloured with blood pigment.

Fig.18 shows a single colony of a 3-day blood agar culture of T238. Note the smooth surface, the entire edge and the zone of haemolysis, with the individual red cells clearly visible, only outside the zone. The surface of the colony is in a different plane from that of the medium and so both cannot be exactly in focus, using such a large degree of magnification. The translucence of the colony made it very difficult to attain any degree of contrast, especially in view of the light-diffusion caused by the blood in the medium.

Appearance of growth in fluid media.

The best medium for this is 1% sugar plasma digest broth, inoculated as described in Chapter 5, Section (1) Method (2). After 24 hours aerobic incubation a whitish haze is seen on the bottom of the tube and the indicator is just turning from greenish to yellowish. When the tube is shaken a faint turbidity, just distinguishable above that of the plasma, is seen.

After 48 hours incubation, definite whitish discrete colonies are visible on the bottom of the

tube, covering this completely. There is very little increase in turbidity of the supernatant fluid until the tube is shaken, when the colonies are emulsified gradually and become suspended evenly to give a turbidity around Brown's opacity 3 or 4.* Although the suspension appears at first to be smooth, close examination reveals its rough character and rapid sedimentation occurs, leaving the supernatant almost clear. The colour of the indicator is now definitely yellow. (Fig. 30).

Little further increase in the amount of growth occurs on further incubation, but, where growth has been poor, the indicator may take several days to change colour. Rarely, a tube showing a negative reaction at 5 days may show a positive after 12 days incubation.

Appearance of growth in thioglycollate medium:

Only a few strains were grown in this medium, with or without the addition of plasma. The illustrations (Figs. 26-29) show the type of growth obtained. Most strains grew equally well at all levels in the tube, but some showed a definite anaerobic tendency, e.g. T238, growth of which suddenly stops short at the edge of the aerobic zone, as indicated by the pink colour of the resazurin dye used in Oxoid fluid thioglycollate medium. (v. Appendix No. 64). Very poor growth,

* v. Brown (1919).

not carried on in successive sub-cultures, was all that was possible in Oxoid fluid thioglycollate medium without plasma, but heavy growth occurred when 5% plasma was added, and also in plain thioglycollate medium without eH indicator (v. Appendix numbers 61 and 62). When dilute inocula were used (0.2 ml. of Brown's opacity 1/1000) separate colonies appeared after 48 hours incubation, as minute white dots, which rapidly grew until the puff-ball appearance described by Gardner and Dukes (1955) was seen at 3 days. This appearance was quite striking as is seen in Figs. 27 & 28.

Section 3. Haemolytic properties of HILB:

Methods.

i. Blood agar was used as already described. This was the easiest and most reliable way of detecting haemolysis. Plates were prepared, using blood of different species of animals and inoculation was made by dropping serial 10-fold dilutions of 48-hour blood agar cultures on to the surface. Results were read after 48 and 72 hours incubation.

ii. Tube tests. Great difficulty was experienced in performing these and variable results were obtained, including many failures to demonstrate

haemolysis at all.

Tube method 1:

Tubes of plasma digest broth (Appendix No.35) were inoculated with a 48 hours blood agar culture of each strain and incubated 48 hours aerobically at 37° C. The culture was then divided into samples and treated as follows:-

Tube a. Centrifuged and supernatant collected. The deposit from this tube was washed x 2 with saline and then made up to the original volume of culture, with saline.

Tube b. Boiled for 5 minutes.

Tube c. Used as live culture.

To 1 ml. volumes of above, 2 drops of 20% suspension of washed and packed human red cells were added and the tube shaken, incubated 2 hours at 37° C. in a water-bath, read, and allowed to stand at room temperature overnight.

Tube method 2:

Blood agar half-plates were inoculated in duplicate from a 48 hour blood agar culture and incubated 48 hours aerobically at 37° C. The resultant growths were then scraped off into 0.8ml. sterile human plasma. Sugar fermentation tubes (as described in Chapter 5, section 1, and Appendix No. 37) containing maltose or arabinose (whichever sugar the strain was known to ferment) were each

inoculated with 0.15 ml. of the plasma/HILB emulsion. After 48 hours incubation at 37° C., the 3 tubes of culture of each strain were pooled, centrifuged and the deposit made up with 2% peptone broth to 3 ml. This gave a dense suspension of HILB. The pH was checked and found to be 7.0. The suspensions, in volumes of 0.5 ml., were pipetted into sterile agglutination tubes (3" x ⅜") and 1 drop of 10% washed and packed red cells was added. After shaking, the tubes were incubated in the 37° C. water-bath for 2 hours and haemolysis read. The tubes were then shaken and re-incubated for a further 4 hours, read again, and finally left overnight in the water-bath before the final reading. In the case of the human red cells, the concentration of red cells proved too weak for easy reading, because of the great density of bacterial suspension, so a further drop of 10% red cells was added after 2 hours incubation.

Results:

The results obtained by tube method 1 are given in Table 13.

The strains used in Table 13 were old ones (i.e. prior to the main series of T numbers) and these results could not be repeated owing to the variability of growth in the medium used. It would appear, however, that the haemolysin is

destroyed by boiling and is present both in the supernatant and on the body of the organism.

Table 13.

Strain	Haemolysis using:				
	Live culture	Super-natant	Boiled cult.	Washed susp.	Control
693	++	++	-	+	-
623	+++	+++	-	++	
839	+++	+++	-	- o.r.t. +	
880	+	?+	-	- +	
239	+++	+++	-	± +	
837	++	++	-	+	

o.r.t. = overnight at room temperature.

The results obtained by tube method 2 are given in Table 14, which also shows the results obtained by the use of blood agar plates. Haemolysis of human red cells was observed by the tube method with T30, T38, T104 and 693. Haemolysis of horse cells by the tube method was only observed with strain T104, which showed results as strong as those with human cells.

Using blood agar plates, definite haemolysis was observed with both human and horse cells with strains T30, T38, T104, 693 and T46. T91 only

TABLE 14.

Haemolysis in tube and on plate.

HILB Strain	Red Cell species									
	Human		Horse		Ox		Sheep		Rabbit	
	BA	Tubes (method ii)	BA	Tubes (method ii)	BA	Tubes (method ii)	BA	Tubes (method ii)	BA	Tubes (method ii)
T 30	48 hrs. ++ 72 hrs. ++	2 hrs. − 6 hrs. + 24 hrs. +	+ +	− − −	− −	− − −	− −	− − −	± ±	− − −
T 38	++ ++	− + +	+ +	− − −	− −	− − −	− −	− − −	± ±	− − −
T104	+++ +++	+ +++ +++	+ +	+ +++ +++	− −	− − −	− −	− − −	± ±	− − −
693	++ ++	− + +	+ +	− − −	− −	− − −	− −	− − −	± ±	− − −
T46	+ +		+ +		− −		− −		± ±	
T 91	++ ++		− −		− −		− −		± ±	
Control broth		− − −		− − −		− − −		− − −		− − −

+++ = marked haemolysis (complete in the case of tube tests).
++ = moderate haemolysis (a button of cells still left in the case of tube tests).
\+ = slight haemolysis (supernatant tinged pink in the case of tube tests).
BA = Blood agar.
± = indicates a very faint, ill-defined zone, which appeared in rabbit blood agar shortly before complete lysis of the whole plate took place.

showed haemolysis with human cells. Rabbit blood agar gave indefinite results, due to spontaneous lysis occurring after two to three days. These results are expressed as ± in table 14. Ox and sheep cells showed no lysis at all by either method. Human blood gives much better zones of haemolysis than horse blood when used in blood agar.

Section 4. Effect of temperature and atmosphere:

Method:

Small portions of blood agar plates were heavily inoculated from 48-hour blood agar cultures. Each strain was cultured (1) at room temperature, (2) at 37° C. aerobically, (3) at 42° C and (4) at 37° C. with 10% CO_2 added. CO_2 was added by evacuating the jar with the water pump until the pressure had been reduced by 3" of mercury, closing the inlet tap and connecting it to a CO_2 cylinder and bladder from which air had been expelled by partially filling the bladder with CO_2 and emptying it. The tap was then opened and CO_2 allowed to enter until atmospheric pressure was restored in the jar. This gave approximately 10% CO_2 in the jar atmosphere.

Cultures were read after 48 hours and after 72 hours incubation, the amount of growth being assessed simply by the naked eye appearance. At

first aerobic plates were incubated in the usual way in the incubator, but then it was realised that the drying which occurs under normal conditions might affect the growth as compared with the CO_2 plates, which were, of course, incubated in a sealed jar. Accordingly, as is indicated in Table 15, the strains from T102 onwards, were incubated in a sealed jar similar to the one containing the CO_2.

Results:

These are given in Table 15. It can be seen that no growth occurred either at room temperature or at 42° C., but that growth was similar at 37° C. with or without CO_2. Strains showing slight improvement of growth due to CO_2 (growth increased by + after 72 hours, or 48 hours where no 72 hour reading is available) included the following:

(1) Aerobic plates not incubated in a sealed jar:- T numbers 7, 10, 30, 38, 60, 88, 98, 100 and 104.

(2) Aerobic plates incubated in a sealed jar:- T numbers 115, 121, 124, 126, 162, 168 and 169.

Strains showing a marked improvement of growth due to CO_2 (growth increased by ++ or more after 72 hours, or 48 hours where no 72 hour reading is available) included:

(1) Aerobic plates not in jar, T35, T109, T111, T129.

(2) Aerobic plates in sealed jar - T145.

TABLE 15.

A.

Growth at Room Temperature 37° C., 37° C. + 10% CO_2 and 42° C.

Aerobic plates not in sealed jars:

T number of Strain	R.T.		37° C.		37° C.+ 10% CO_2		42° C.	
	48 hrs.	72 hrs.	48 hrs.	72 hrs.	48 hrs.	72 hrs.	48 hrs.	72 hrs.
7	-	-	++	•	+++	•	-	-
10	-	-	+	++	++	+++	-	-
12	-	-	++	•	++	•	-	-
15	-	-	++	•	++	•	-	-
20	-	-	+++	+++	+++	+++	-	-
30	-	-	+	•	++	•	-	-
33	-	-	++	++	++	++	-	-
34	-	-	++	++	++	++	-	-
35	-	-	+	+	++	+++	-	-
38	-	-	+	+	++	++	-	-
46	-	-	+++	+++	+++	+++	-	-
60	-	-	-	-	+	+	-	-
62	-	-	++	++	++	++	-	-
66	-	-	+++	+++	+++	+++	-	-
73	-	-	+	+	+	+	-	-
83	-	-	+++	+++	+++	+++	-	-
86	-	-	+	+	+	++	-	-
87	-	-	++	++	++	++	-	-
88	-	-	±	-	+	+	-	-
91	-	-	++	++	++	++	-	-
92	-	-	++	+++	+++	+++	-	-
94	-	-	++	+++	+++	+++	-	-
98	-	-	++	++	+++	+++	-	-
100	-	-	+	++	++	+++	-	-
104	-	-	+	++	+++	+++	-	-
109	-	-	+	+	+++	+++	-	-
111	-	-	+	+	++	+++	-	-
129	-	-	+	+	++	+++	-	-

TABLE 15 (contd.).

B.

Growth at Room Temperature 37° C., 37° C. + 10% CO_2 and 42° C.

37° C. Aerobic plates incubated in jars to prevent drying:

T number of Strain	R.T.		37° C.		37° C. + 10% CO_2.		42° C.	
	48 hrs.	72 hrs.	48 hrs.	72 hrs.	48 hrs.	72 hrs.	48 hrs.	72 hrs.
102	-	-	+++	+++	+++	+++	•	•
104	-	-	+++	+++	+++	+++	-	-
115	-	-	++	++	++	+++	-	-
121	-	-	+	+	++	++	-	-
124	-	-	+	•	++	++	-	-
126	-	-	+	•	++	•	-	-
131	-	-	+++	+++	+++	+++	•	•
132	-	-	+++	+++	+++	+++	•	•
140	-	-	+++	+++	+++	+++	•	•
145	-	-	+	+	++	+++	•	•
147	-	-	++	+++	+++	+++	-	-
149	-	-	++	++	++	++	-	-
162	-	-	++	++	+++	+++	-	-
168	-	-	+	++	++	+++	-	-
169	-	-	+	++	+++	+++	-	-

R.T. = room temperature.

- = no growth.

+ = scanty growth.

++ = moderate growth.

+++ = heavy growth.

• = plates not readable, due to contamination.

It will be seen that nine out of twenty-eight (32%) of the series where aerobic plates were not incubated in a sealed jar, showed a "+" improvement in growth, in the CO_2 cultures. Of the fifteen in the series, with aerobic plates incubated in a sealed jar, seven showed a "+" increase in growth due to CO_2 (50%). The effect of failing to seal the aerobic plates in a jar was therefore unimportant. The number showing a marked improvement in growth (++ or more) was four out of twenty-eight (14%) where the aerobic controls were not sealed, but only one out of fifteen (7%) where the controls were sealed. The effect of sealing the jar seemed therefore more important than the CO_2 in the case of strains showing a marked improvement in growth. In spite of this, the fact remains that the addition of CO_2 caused a slight improvement in growth in 50% of properly controlled cases and a marked improvement in 7%.

Effect of anaerobiosis:

No special series was carried out to show this on blood agar, but all incoming specimens for HILB isolation were invariably incubated both aerobically and anaerobically. Sometimes the aerobic plate alone yielded HILB and sometimes (more frequently) the anaerobic alone. Usually both aerobic and anaerobic cultures gave a successful result in

primary isolation, although contaminants tended to be smaller on the anaerobic plates, thus rendering identification and picking of the colonies easier.

Secondary cultures have always proved to be able to grow both aerobically and anaerobically with approximately equal facility. Since strain T238 can grow well aerobically on blood agar, it is surprising that it shows such a definite predilection for anaerobic conditions when grown in plasma thioglycollate medium, especially since the predilection is not at all affected by adding an excess of catalase to the medium. None of the thioglycollate cultures have shown any evidence of a microaerophilic tendency (except for T94 in Oxoid fluid thioglycollate).

Anaerobic conditions have enabled scanty growth, not surviving in sub-culture, to occur on 5% digest agar, when 50% digest broth was used to dilute the inoculum. This growth was improved when cholesterol, lecithin or cephalin was included in a concentration of 1/100,000 and, in the case of cholesterol, growth continued for 2 successive sub-cultures of 2 strains, 3 of 1 strain and 4 at least of a 4th strain. No growth occurred in any of these media when incubated aerobically. Growth on 5% serum digest agar was also markedly improved by anaerobic incubation, although scanty growth also

occurred aerobically.

Anaerobic cultivation in the presence of 14% CO_2 gave better results, using media other than blood agar, than either aerobic or anaerobic culture, and even blood agar cultures were noticeably improved by this method. Growth was not, however, obtained in ordinary broth or 5% digest broth by the use of anaerobiosis plus CO_2, and growth in plasma digest broth and serum digest broth was little improved by it.

Section 5: Viability, and effect of pH on growth:

Methods:

i. Thermal death point measurement:

Serial dilutions of strain T216 were made in 2% peptone broth as follows. A 48-hour blood agar culture was emulsified in 0.2 ml. broth until the opacity was approximately Brown's no.1 (Brown,1919). Broth was then added to give a 1/10 dilution. 0.5 ml. volumes were then carefully put at the foot of 5"x½" tubes so as to avoid wetting the sides of the tube. They were then placed in water-baths kept at 40, 50 or 60°C. for 10 minutes, with 1 tube as control at room temperature. After 10 minutes, the tubes were removed and cooled. Three further 10-fold serial dilutions were made in broth and 1 drop of each added to each of 2 blood agar plates, The range

of dilutions was thus from opacity 1/10 to 1/10,000. The plates were then incubated 48 hours, readings made and re-incubation carried out for a further 24 hours.

ii. Effect of drying:

Filter paper (Whatman No.1) disks, 6.5 mm. in diameter, were sterilised by hot air and used for this experiment. Eight disks were soaked in opacity 1 dilution of T.216 culture, prepared as above, this time in plasma digest broth (Appendix No. 35). Each disk was then laid separately in a Petri dish, which was placed in a desiccator over P_2O_5 at room temperature, with the Petri dish lid partially removed, to allow rapid drying of the disks. After 2 hours drying, 2 disks were removed, and soaked in 0.2 ml. peptone broth. As each disk absorbs approximately 0.01 ml. (Gould and Bowie, 1952) this gave roughly a 1/10 dilution of the original opacity 1 suspension. Three 10-fold serial dilutions were then made from this as before, and inoculated in the same manner on 2 blood agar plates. The procedure was repeated after 4, 6 and 24 hours. Controls consisted of disks kept at room temperature in a Petri dish, sealed, along with a moist cotton wool pad, in a jar. They were processed at the same intervals as the test disks,

at the same time and in the same way.

iii. <u>Effect of cold:</u>

An opacity 1/10 dilution was made of T216, in plasma digest broth, divided into 0.5 ml. quantities and placed in the refrigerator. One tube was removed at intervals of 4, 24, and 48 hours. The same procedure as above was then carried out to inoculate these suspensions on blood agar. Controls consisted of 0.5 ml. tubes of the same suspension as the test ones, but kept at room temperature and sampled at the same time as the refrigerated tubes.

iv. <u>Effect of pH on growth:</u>

To 2% peptone broth (Appendix No. 1), 5% human plasma was added and samples of the medium adjusted by adding N/10 HCl or N/10 NaOH to the following pH levels: 4.5, 5.5, 6.5, 7.0, 7.5 and 8.5. The amount of acid or alkali to be added to a given volume was noted.

Fresh batches of medium were then made up, using the appropriate amount of acid or alkali already determined for each of the above pH levels, but with no added plasma. The medium was then steamed for the usual time and 1% maltose added aseptically from 20% sterile solution. To a separate batch, 0.25% maltose was added. Three ml. volumes were tubed aseptically into 5" x 5/8" tubes and steamed for 10 minutes. The medium was

now ready for use. Sugar fermentation method 2 (v. Chapter V, Section 1) was used for inoculation (0.15 ml. HILB/plasma emulsion added to 3 ml. medium). Controls were "inoculated" with plasma but no organisms, and their pH measured by B.D.H. capillator. The results of this manoeuvre are seen below.

pH of original test mixtures	pH after steaming and adding sugar and plasma.	
	1% maltose	0.25% maltose
4.5	4.6	4.7
5.5	5.6	5.7
6.5	6.0	6.2
7.0	6.5	6.5
7.5	7.0	7.0
8.5	8.0	8.0

The pH levels were not exactly as intended after the plasma was added, but the range proved quite suitable for estimating the effect of pH on growth.

After the inoculated tubes had been incubated for 48 hours, the amount of growth was noted, and sub-cultures carried out by Pasteur pipette, 1 drop from each tube being placed on the surface of a blood agar plate.

Results:

i. Thermal death point: Results are seen in Table 16.

Table 16.

Effect of heating at various temperatures for 10 minutes.

Exposure Temperature	Incubation period (hours)	Growth at various dilutions*				Colony size
		10	10^2	10^3	10^4	
40° C.	48	C	AC	>20	>20	Medium
	72	C	AC	>20	>20	
50° C.	48	>20	<20	0	0	Small
	72	>20	<20	0	0	
60° C.	48	0	0	0	0	
	72	0	0	0	0	
Control (R.T.)	48	C	AC	>20	>20	Medium
	72	C	AC	>20	>20	

* = Reciprocal of fraction of dilution of Brown's opacity 1. C = Confluent; AC = almost confluent; >20 = more than 20 colonies; <20 = less than 20 colonies; R.T. = room temperature.

It will be seen that the number of viable particles in the suspension exposed to 40° C. for 10 minutes was the same as in the control. Expo-

sure to 50° C., however, caused a reduction of over 99% in the number of viable particles. Exposure to 60° C. caused the death of over 99.99% of particles present. The thermal death point can therefore be given as between 50° C. and 60°C. for an exposure of 10 minutes in peptone broth.

ii. Effect of drying:

This is seen in Table 17.

Table 17. Effect of drying at room temperature.

Time of Exposure	Growth, after 72 hours incubation, of various dilutions.				Colony size
	10	10^2	10^3	10^4	
0 (control)	C	AC	>20	<20	medium
2 hours	< 20	0	0	0	very small
2 hours control	AC	>20	> 20	< 20	medium
4 hours*	< 20	0	0	0	very small
4 hours * control	AC	>20	<20	< 20	small
6 hours	0	0	0	0	
6 hours control	AC	>20	>20	< 20	small
24 hours	0	0	0	0	
24 hours control	0	0	0	0	

* 1 plate only could be read. Except for these, the figures represent the average of 2 plates. Abbreviations as in Table 16.

It will be seen that 2 hours drying under the conditions of the test, led to 99.9% reduction in the number of viable particles. The remaining particles showed reduced vigour as shown by the smaller size of the colonies as compared with the control. Six hours drying led to over 99.99% killing. Exposure of the control disks to 6 hours at room temperature in moist conditions in the favourable medium used, did not reduce the viability of the organisms to any appreciable extent, but keeping the disks for 24 hours under these conditions led to at least 99.99% killing.

iii. Effect of cold. See Table 18.

Table 18. Effect of refrigeration.

Time of Exposure	Time of incubation (hours)	Growth at various dilutions			
		10	10^2	10^3	10^4
4 hours	48	AC	>20	>20	<20
	72	AC	>20	>20	<20
4 hours control.	48	C	AC	>20	>20
	72	C	AC	>20	>20
24 hours	48	>20	<20	0	0
	72	>20	<20	0	0
24 hours control.	48	>20	<20	0	0
	72	>20	<20	0	0
48 hours	48	>20	0	0	0
	72	>20	<20	0	0
48 hours control.	48	>20	0	0	0

Abbreviations as in previous table.

The results given in Table 18 show that there is no difference in the mortality rate of the test strain when kept at 4° C. in plasma broth from that of a similar specimen kept at room temperature.

iv. Effect of pH on growth:

The results are seen in Tables 19, and 20.

The amount of growth is seen from Table 19 to be maximal at pH 6.0 - 6.5, and minimal at pH 4.5. No growth at all occurred at pH 8.0.

The viability at different pH levels is seen in Table 20. This shows that survival at low pH levels is good, all 3 uncontaminated strains giving growths from the pH 4.5 tubes, both from 1% and 0.25% sugar concentrations. Failure to sub-culture occurred at all other pH levels, except in some of the tubes showing + or ++ growth, e.g., T46 from pH 6.0 and 7.0 (1%) tubes. No tube showing a +++ growth yielded a successful sub-culture. It seems therefore that survival in maltose plasma broth depends more on the amount of growth than on the pH, the heavy growths being self-sterilising while the scantier growths occurring at low pH (or occasionally at high pH) levels remained viable during the 48-hour period of the experiment.

Further information on the effect of pH was obtained when an attempt was made to make acid production in sugar media more clearly visible,

TABLE 19.

Effect of pH on growth.

Strains:	Growth in 1% Maltose broth						Growth in 0.25% Maltose broth					
	pH						pH					
	4.5	5.5	6.0	6.5	7.0	8.0	4.5	5.5	6.0	6.5	7.0	8.0
T30	+	++	+++	+++	+++	-	+	++	+++	+++	++	-
T46	+	++	++	+++	+	-	+	++	++	+++	+	-
T91	+	++	+++	+++	+++	-	+	++	+++	+++	++	-
T94	+	++	++	+++	+++	-	+	++	++	++	++	-
Control (no organisms)	-	-	-	-	-	-	-	-	-	-	-	-

\+ = Growth giving Brown's opacity 2 after shaking the tubes, i.e. scanty growth

+++ = Growth giving Brown's opacity 7-8 after shaking the tube - heavy growth.

++ = Intermediate between + and +++

\- = No visible growth.

TABLE 20.

Viability at different pH levels.

Strains:	Subculture from above tubes on blood agar, incubated 5 days.											
	Original pH of tube											
	1% maltose plasma broth.						0.25% maltose plasma broth					
	4.5	5.5	6.0	6.5	7.0	8.0	4.5	5.5	6.0	6.5	7.0	8.0
T30	+	-	-	-	-	-	+	-	-	-	-	-
T46	+++	-	++	-	+++	-	+	-	-	-	-	-
T91	+++	-	-	-	-	-	+++	+	-	-	-	-
T94	Subculture contaminated.											

by raising the pH from 7.4 to 7.6. This made the colour more blue and it was hoped that this would accentuate any change to yellow resulting from acid production. Growth of HILB was, however, very much poorer in this medium, so that it appears that pH 7.6 represents the upper limit for growth.

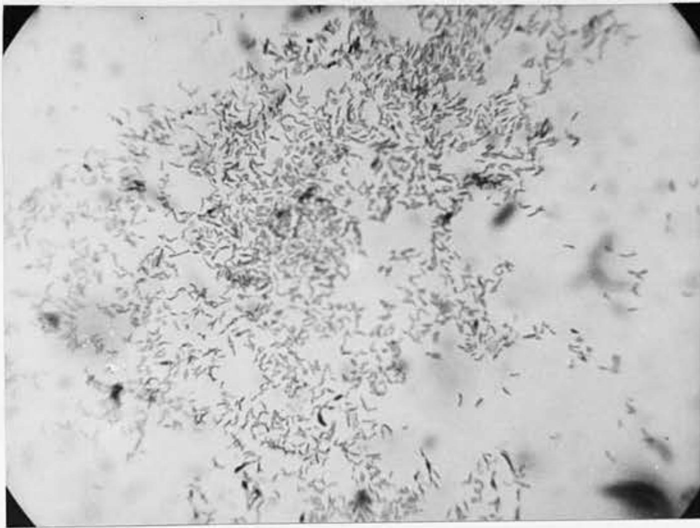

Fig. 4. Smear from high vaginal swab, case of puerperal pyrexia (HILB isolated +++), showing numerous small Gram negative bacilli.
Stained Gram. X 750

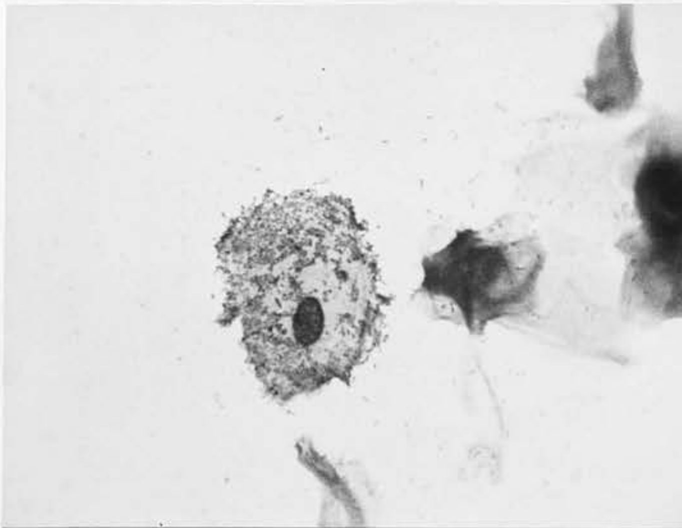

Fig. 5. Smear from high vaginal swab from case of puerperal pyrexia, showing HILB adhering to an epithelial cell in large numbers. So-called "clue-cell"of Gardner and Dukes (1955).
Stained Gram. X 875

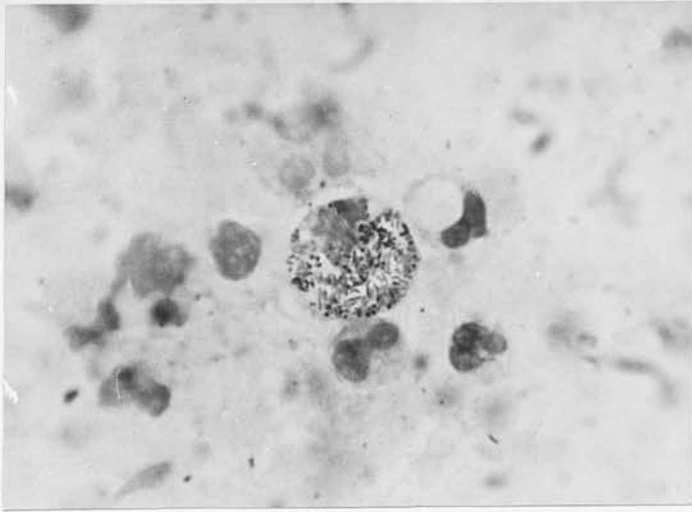

Fig. 6. Smear from high vaginal swab, case of puerperal pyrexia (HILB isolated +++), showing leucocyte packed with HILB.

Stained Gram. X 900

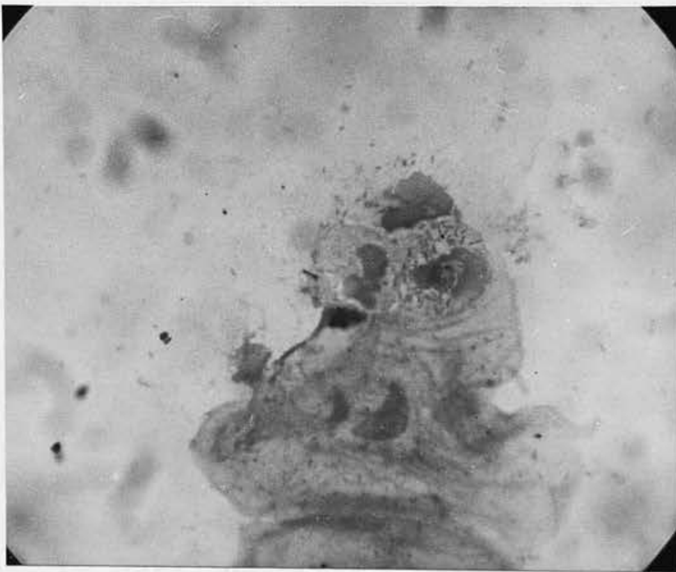

Fig. 7. Smear from similar specimen to that shown in Fig. 6 , showing numerous intra-cellular HILB.

Stained Gram X 750

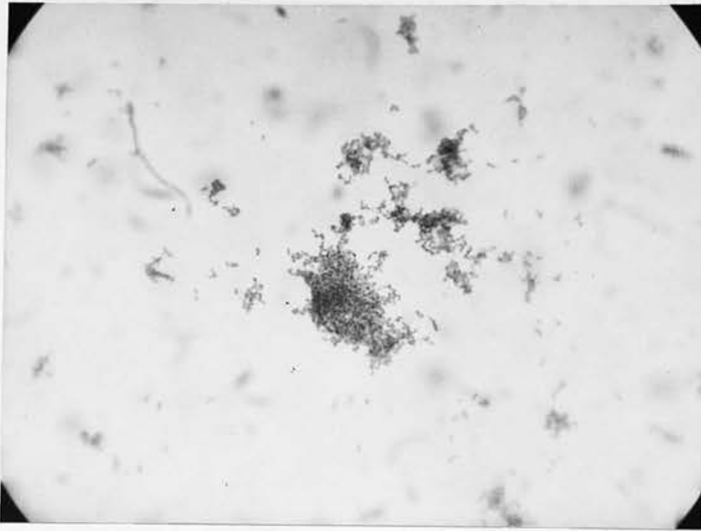

Fig. 8 48-hour blood agar culture of T238.
Stained Gram. X 750.

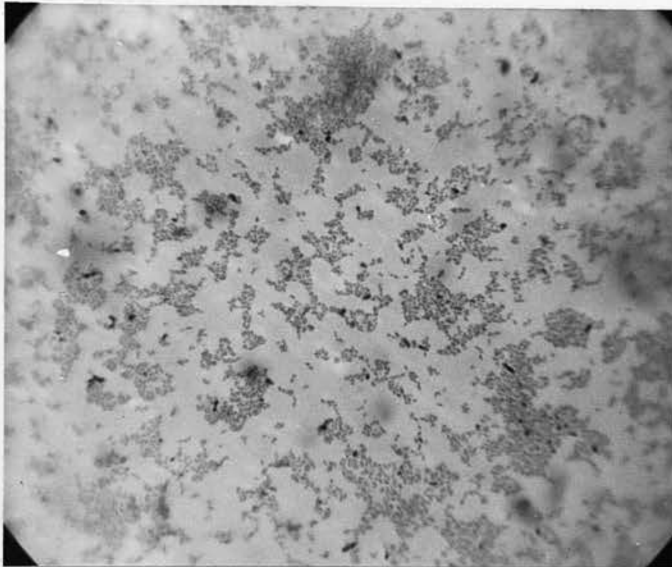

Fig. 9 48-hour anaerobic blood agar culture of T238, showing minute cocco-bacilli, with a few Gram positive granules.
Gram's stain. X 750.

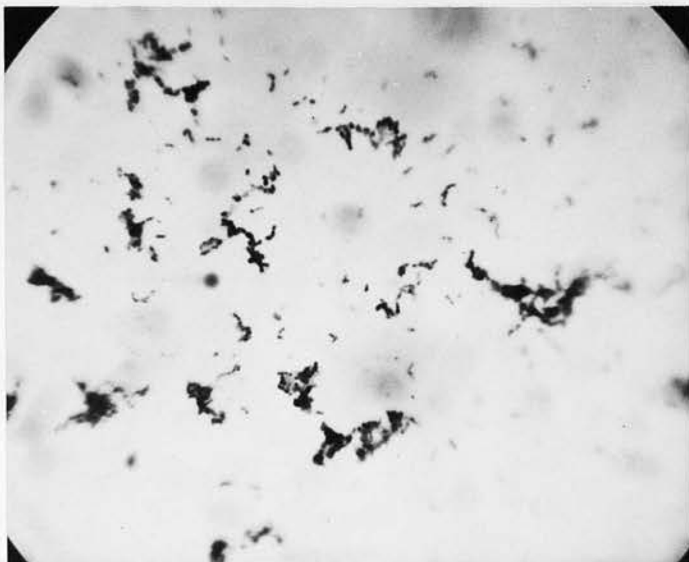

Fig. 10. 48-hour aerobic culture of T238 in plasma digest broth, showing the beaded Gram positive appearance thus induced.

Stained Gram. X 750.

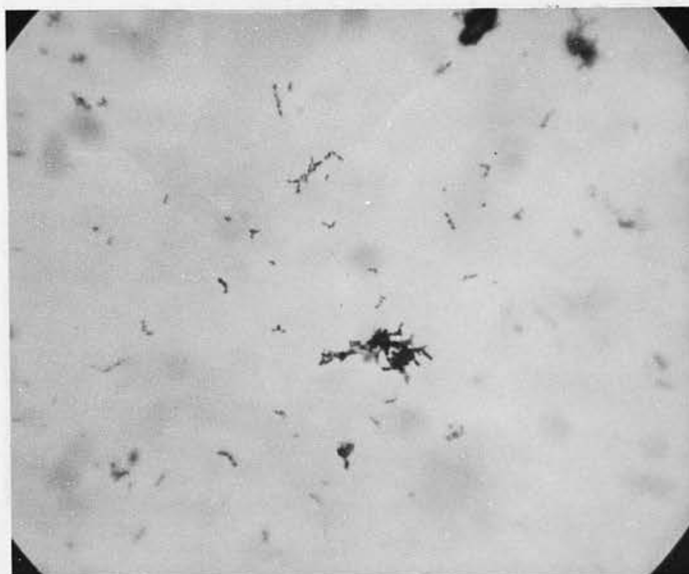

Fig. 11. 48-hour anaerobic culture of T238 in PDB, showing Gram positive, beaded appearance, less marked than in aerobic fluid culture.

Stained Gram. X 750.

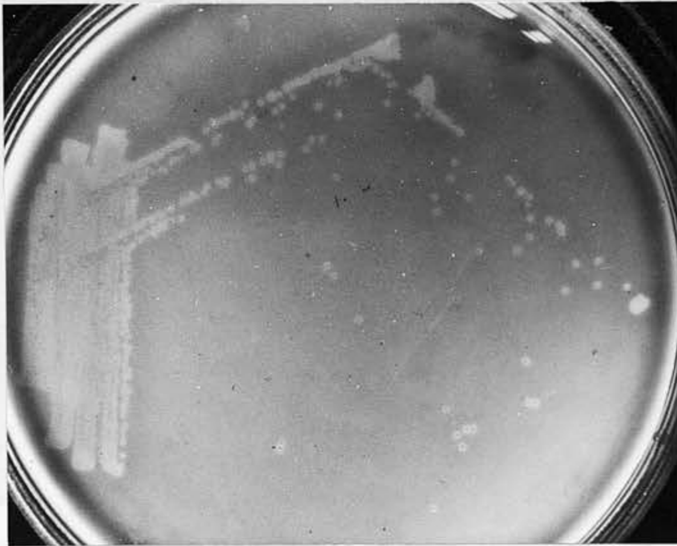

Fig. 14. 3-day blood agar culture of T238, actual size.

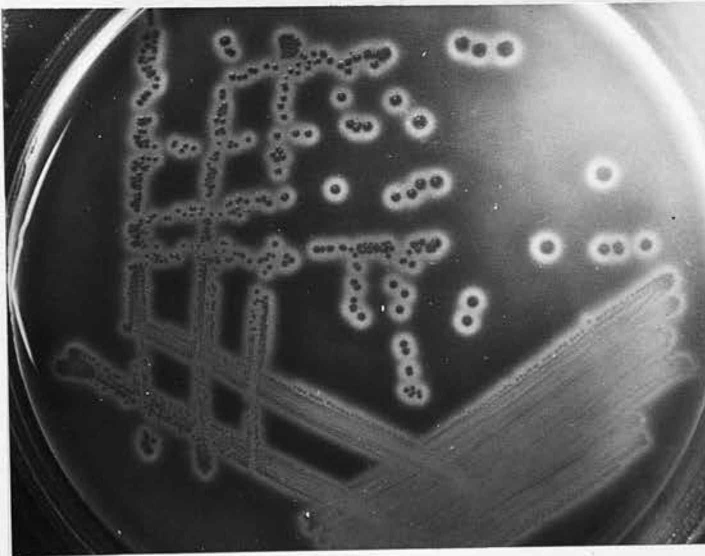

Fig. 15. 3-day culture of <u>Strep. pyogenes</u> on blood agar, showing the very much larger size of colony compared with HILB (v. Fig 14). Actual size.

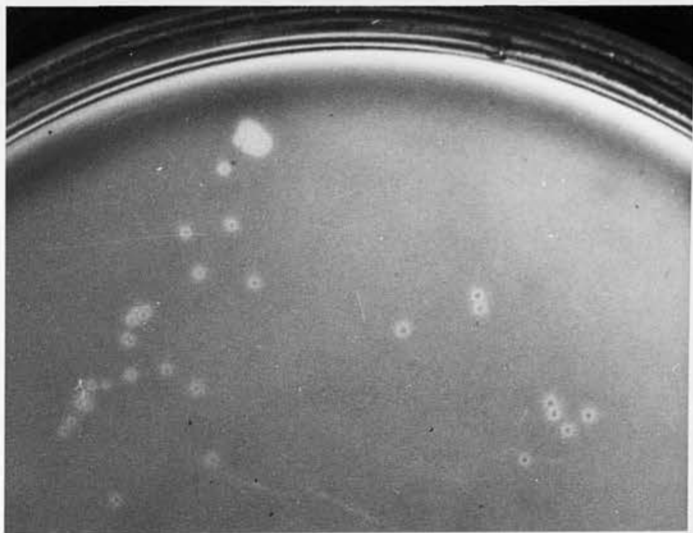

Fig. 16. 3-day blood agar culture of T238, showing haemolysis. X 2.

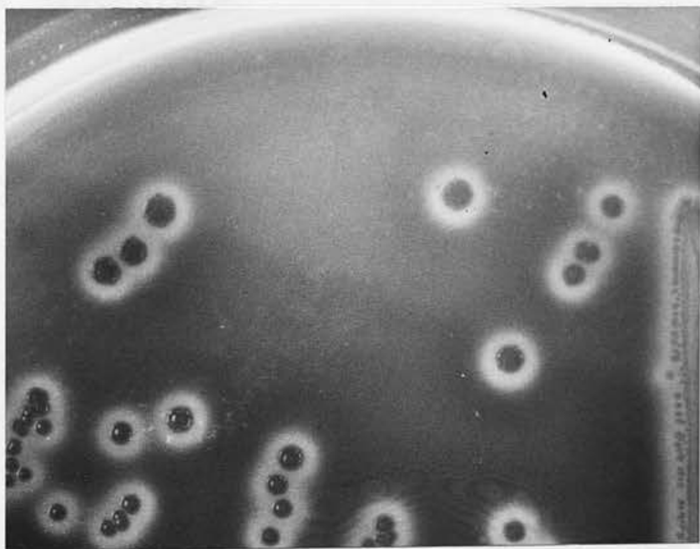

Fig. 17. 3-day blood agar culture of Strep. pyogenes, for comparison with Fig. 16 above, showing the different order of size compared with HILB colonies. X 2.

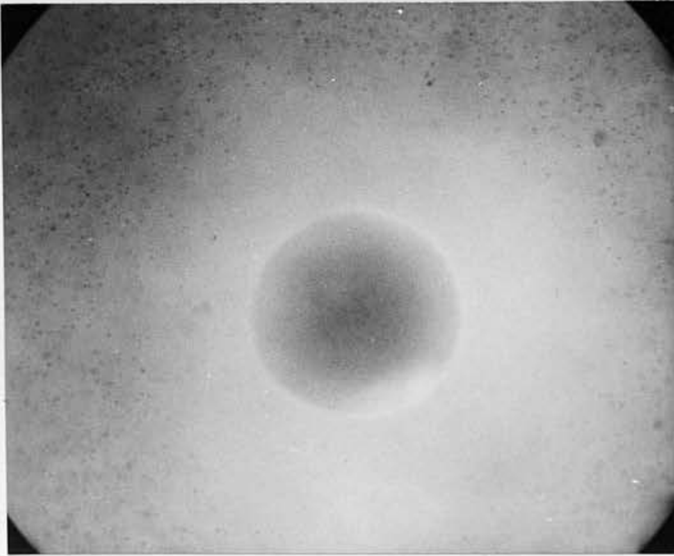

Fig. 18. 3-day aerobic culture of T238 on blood agar. Single colony showing smooth, domed, translucent nature and absence of red cells in zone of haemolysis. X 90.

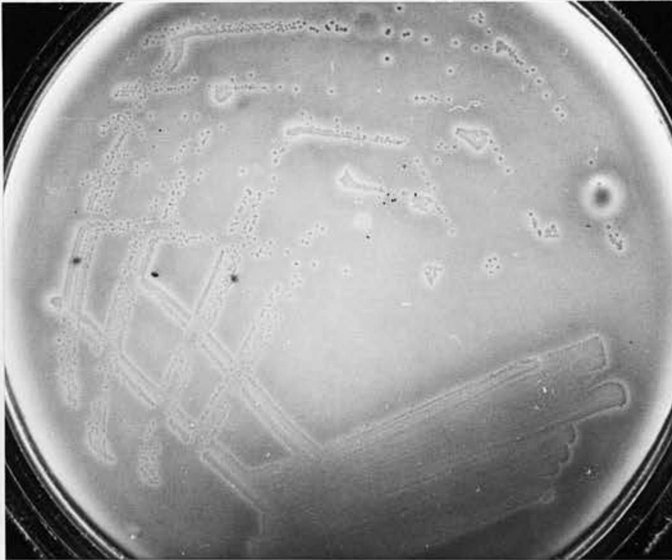

Fig. 19. 48-hour aerobic culture of T238 on 1% maltose blood agar. Actual size. The colonies are larger and more opaque than on blood agar (v. Fig.).

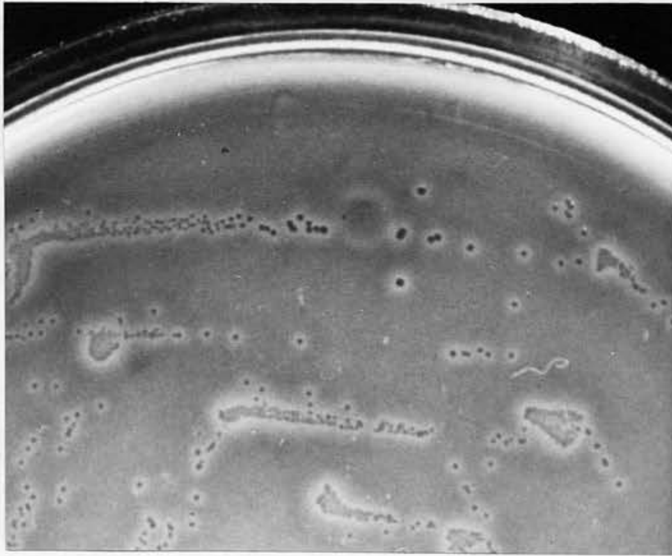

Fig. 20. 48-hour aerobic culture of T238 on 1% maltose blood agar. X 1.75.

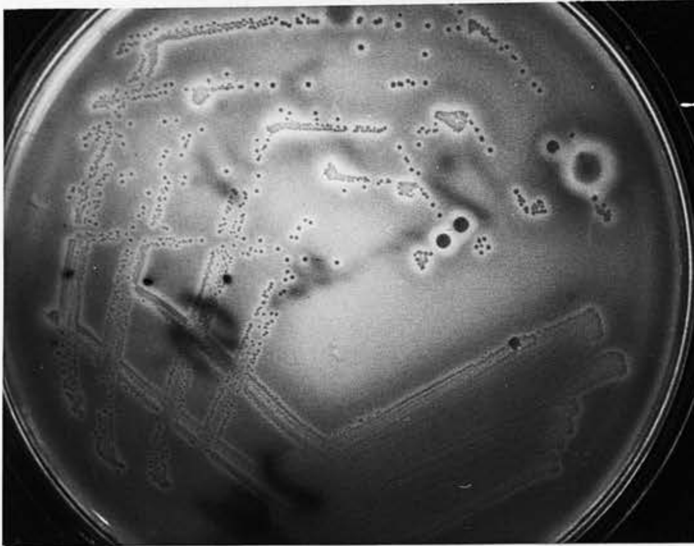

Fig. 21. 3-day culture of T238 on 1% maltose blood agar, viewed by transmitted light only, showing haemolysis and larger colonies than on blood agar. (v. Fig. 14). Actual size.

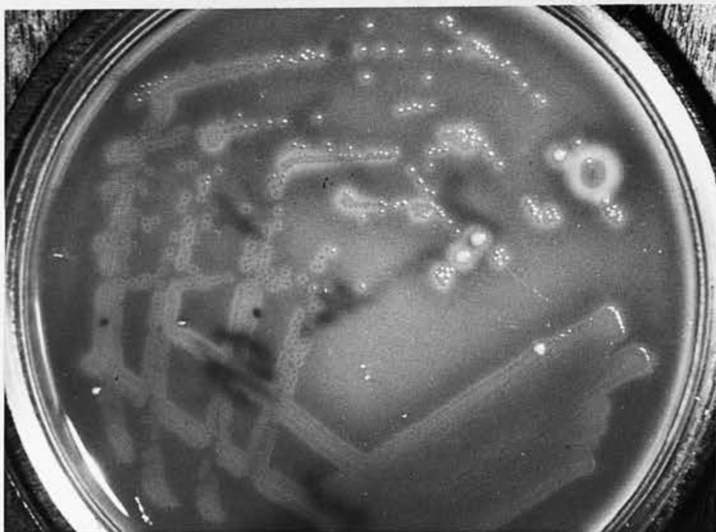

Fig. 22. 3-day aerobic 37°C culture of T238 on 1% maltose blood agar, viewed by both reflected and transmitted light. Colonies are larger than on blood agar, but still very small compared with the contaminating colonies. Actual size.

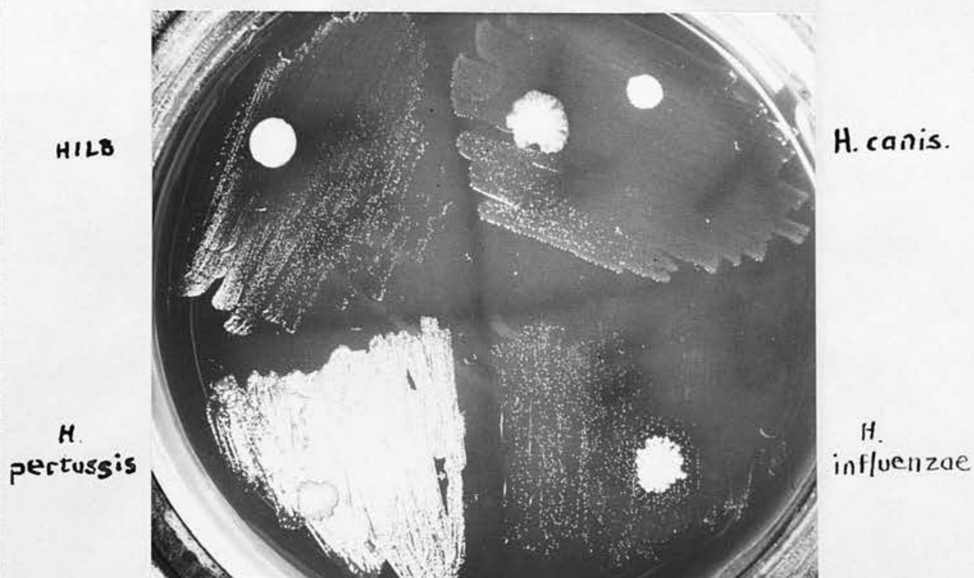

Fig. 23. 48-hour blood agar cultures of HILB, H. canis, H. pertussis and H. influenzae, with pin-point inoculation of staphylococci. Note satellitism of H. influenzae, but not of the other three. Note also the relative luxuriance of growth of H. canis and H. pertussis compared with HILB. Actual size.

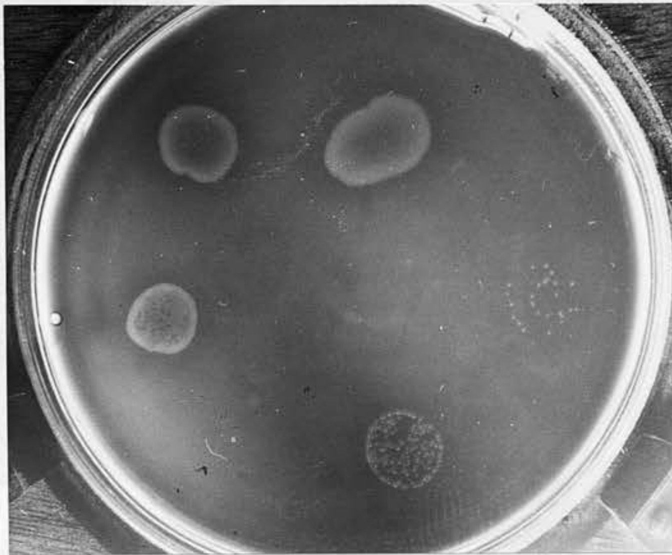

Fig. 24. 3-day culture from serial dilutions of strain T145 on human blood agar, showing haemolysis. Slightly less than actual size.

Fig. 25. 3-day culture of serial dilutions of T145 on autoclaved blood agar. The relatively large size of the contaminating staphylococcus colony can be seen, and the absence of satellitism round it. Slightly less than actual size.

Fig. 26. 48-hour cultures of T145 in thioglycollate broth. Tubes 1 and 2, with 0.15% New Zealand agar, tubes 3 and 4, with 0.05%; tubes 5 and 6 are Oxoid fluid thioglycollate. Inoculum dilutions were Brown's opacity 1/10 in tubes 1, 3 and 5, and 1/1000 in the remainder. X 5/7

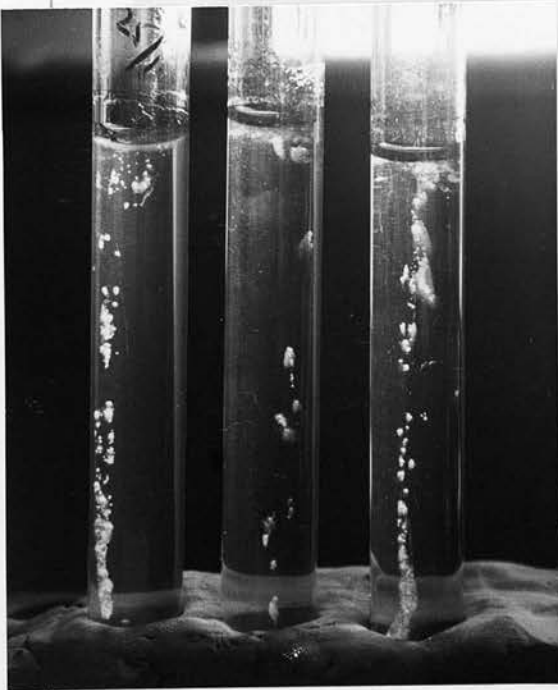

Fig. 27. 48-hour cultures in thioglycollate broth of strain T91, showing puff-ball appearance. Actual size.

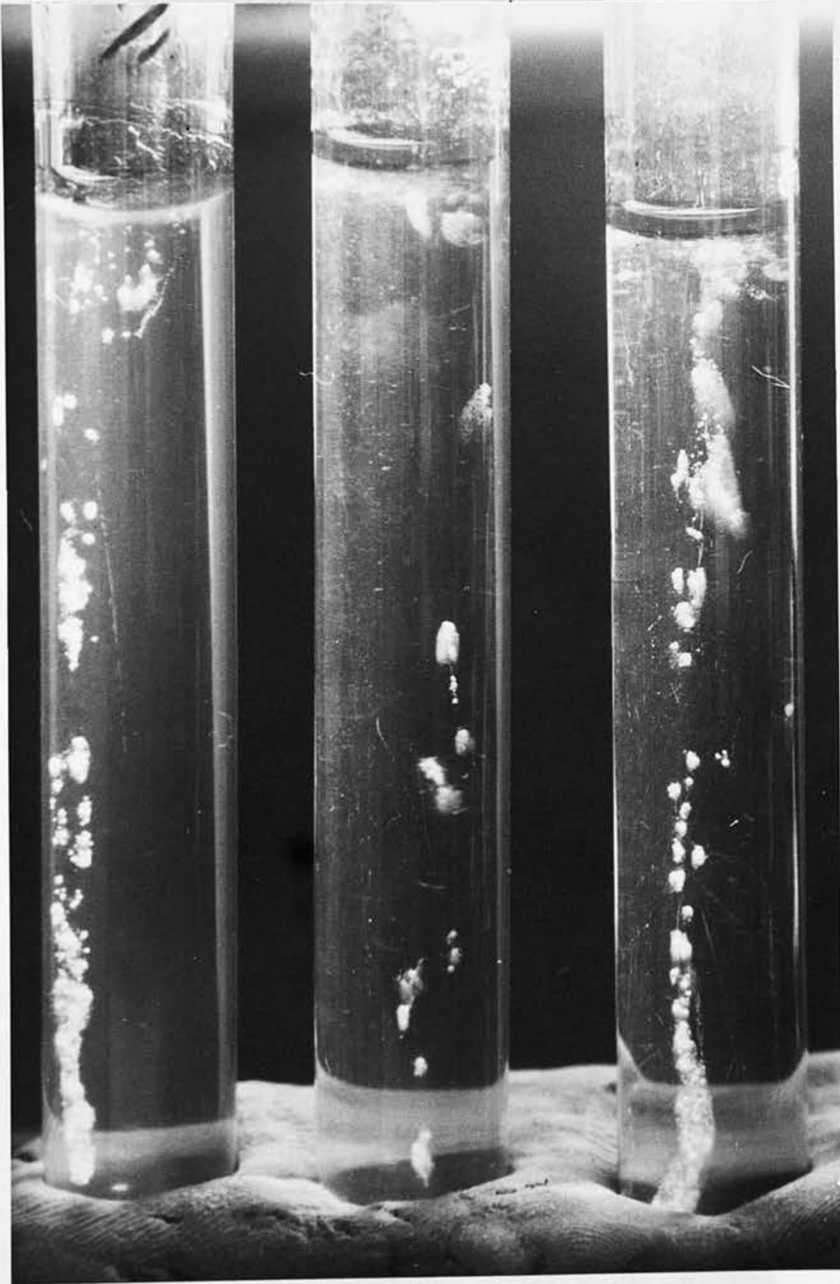

Fig. 28. This is Fig.27. enlarged X 2.

Fig. 29. 48-hour cultures in plasma thioglycollate (Oxoid). L. to R., T145, T183 and T238, each inoculated with Brown's opacity 1/10 and 1/1000 suspension. In tubes 3, 4 and 5 an anaerobic distribution of growth can be seen. X 5/7.

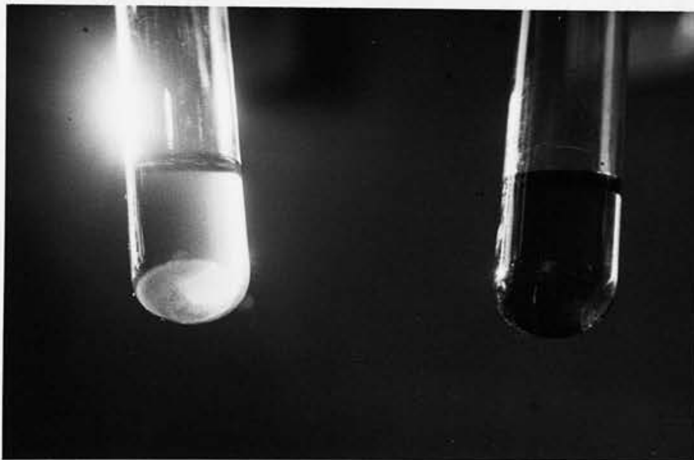

Fig. 30. 48-hour cultures of T94 in 1% glycogen plasma broth with bromthymol blue indicator (left) before shaking, showing the deposit and colour change, compared with the control tube (right).

Actual size.

CHAPTER IV.

GROWTH REQUIREMENTS.

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Section 1. Methods of assessing the value of culture media.

Selected strains of HILB were inoculated on to the medium to be tried out and assessment of growth or failure to grow made by one or more of the following methods.

a. A heavy inoculum was applied, but carry-over of growth factor was minimised by repeated sub-culture on the medium. Growth was assessed by naked eye, with occasional check by filming if contamination was suspected.

b. The inoculum was emulsified in broth to wash growth factor from the bacilli. A small fraction of this emulsion was then used for inoculation. This was used in comparing the effect of X and V factors on HILB and true *H. influenzae* strains.

c. A 48-hour 37°C. aerobic blood agar culture was emulsified in 0.2 ml. peptone broth to Brown's opacity 1. 1.8 ml. broth was then added to give opacity 1/10. Serial 10-fold dilutions were made from this, up to opacity 1/10,000 in peptone broth. Agar plates containing the substances to be tested for growth-promoting properties were then inoculated by adding with a sterile Pasteur pipette 1 drop of each dilution to the plate, so that a progression

of dilutions of inoculum was obtained. Blood agar was invariably included among the media being tested in any given batch, to act as a control.

Each drop gave a disk of growth on the medium (if growth was supported). In at least one of the dilutions, single countable colonies were present and the result of the experiment was then reported by the following abbreviations:-
C = confluent; AC = almost confluent (single colonies just discernible); >20 = more than 20 separate colonies counted; <20 = less than 20 colonies; 0 = no growth.

In addition, the size of the colonies was noted, as occasionally a medium giving growth from the same dilution of inoculum as blood agar yet failed to match blood agar in respect of size of colonies.

d. Where the medium was required for temporary use, e.g. for sugar fermentation or other biochemical tests, all that was necessary was to ascertain if the organism could be made to grow in the medium, or at least survive for a reasonable time. When this was the case, or when preliminary work was being done, the methods a - c were not used. Instead a single culture with heavy inoculum was carried out and a record made either of the naked eye appearance of the growth or of the amount of growth on sub-culture on blood agar.

Section 2: Miscellaneous techniques used in investigation of growth requirements:

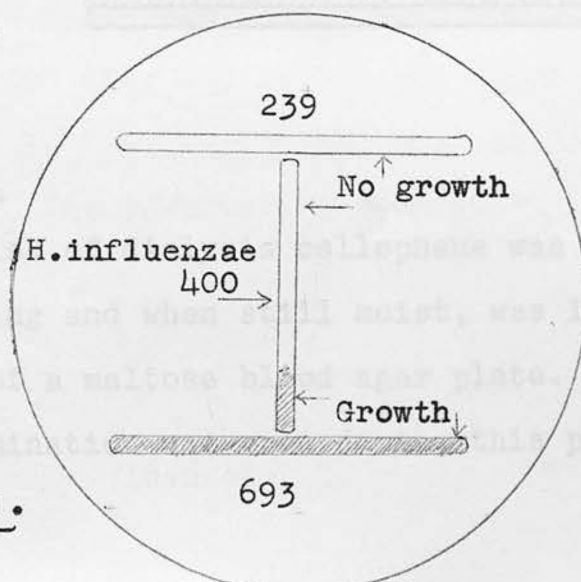
A. Attempts to demonstrate satellitism.

Frequently colonies of staphylococci would contaminate cultures of HILB on blood agar. True satellitism was never seen, although when H. influenzae strains were being used, this was frequently seen. When apparent satellitism was seen on a defective batch of blood agar, efforts made to confirm this showed that the satellitism was due to penicillinase produced by the staphylococcus concerned, which was destroying traces of penicillin in the blood. When

the penicillinase-producing colony was replaced by a penicillin-sensitive strain of staphylococcus, poor growth of this organism (the Oxford standard staphylococcus) occurred and where growth did occur, there was no stimulation of growth of adjacent HILB inocula. Placing a loopful of penicillinase solution on the surface of the HILB-inoculated plate, moreover, caused normal growth to occur, showing that the apparent deficiency in the medium was the fact that penicillin was present (v. Section 3). This was not noticed in routine clinical diagnostic work as the penicillin was insufficient to effect even <u>Strep. pyogenes</u>.

HILB itself can induce satellitism of <u>H. influenzae</u> as seen by the following experiment.

HILB strains 239 and 693 were stroked out as in Fig.31 on human serum agar. 693 grew moderately well, 239 failed to grow. <u>H. influenzae</u> strain 400 was stroked out between the HILB strokes as shown. Growth occurred only near the junction with 693.



<u>Fig. 31.</u>

B. Diffusibility of growth factor.

Method 1.

A thin layer of peptone agar was poured whilst hot on to the surface of a blood agar plate. The volume used was 3 - 4 ml. and this gave a layer of agar 0.5 mm. thick. Growth would not occur immediately on this medium but preliminary incubation of the plate for 1 - 2 days enabled as good growth to occur, as on unlined blood agar. Growth factors were therefore diffusing through the layer of agar. When the agar lining became much thicker, approximately 2 mm., growth ceased completely. This was frequently seen during the preparation of antigens on agar-lined maltose blood agar plates, if the plate had not been level during the lining process or if it was tilted before setting was complete (v Fig.32).

Fig. 32.

Method 2.

A disk of dialysis cellophane was sterilised by steaming and when still moist, was laid on the surface of a maltose blood agar plate. Great risk of contamination occurred during this procedure

and many plates were spoiled. HILB cultures were then streaked on the surface or dropped on as in method c. Growth was observed after several days' incubation. This was scanty and very poor but did occur, with heavy inocula only, in three strains of HILB and one of H. influenzae.

Method 3.

Over the cellophane laid on as in "2" on blood agar a further layer of agar was poured (v. Mazloum and Rowley, 1955). After 2 - 3 days incubation, HILB strains were inoculated heavily, but no growth occurred, even after several days at 37°C.

Section 3: Results of Growth Factor Experiments:

A. Tests with X and V factor, and charcoal.

i. On solid medium:

Using method "a" as defined in Section 1, 3 strains of HILB were tested on blood agar, autoclaved blood agar and autoclaved blood agar plus co-enzyme 1. H. influenzae was included as a control. The results are seen in Table 22.

Table 22 Absence of V factor requirement
(method"a")

Strain		Growth after 3 days 37°C. aerobic		
		Blood agar	Autoclaved* blood agar	Autoclaved blood agar + Co I
HILB	693	+	+	
	966	+	+	
	976	+	+	
H.influenzae		+	-	+

* 30 minutes at 120°C.

Co I = Co-enzyme I, 1 loopful 1/1000.

The above strains were isolated from cases of puerperal pyrexia. All 3 HILB strains grew equally well on blood agar from which V factor had been removed by autoclaving. The absence of V factor was demonstrated by the failure of H. influenzae to grow unless pure co-enzyme I was added. The same conclusion is arrived at by the use of method "c", as seen in Table 23.

Table 23: Absence of V factor requirement (method "c")

		Growth after 3 days 37°C. aerobic															
		Blood agar				Autoclaved blood agar				Autoclaved blood agar + Co I 1/66,000				Serum agar + haematin 1/33,000 + Co I 1/66,000			
Inoculum dilution I		10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4
HILB Strains	T30	C	AC	>20	<20	C	>20	>20	<20	C	C	>20	.	0	0	0	0
	T46	C	AC	>20	>20	C	AC	>20	<20	AC	AC	<20	<20	0	0	0	0
	T91	C	C	>20	>20	C	AC	>20	>20	.	.	.	.	.	.	.	.
	T94	C	AC	>20	>20	AC	>20	>20	.	.	.	.	.	.	.	.	.
	T145	C	AC	>20	>20	AC	AC	>20	.	C	AC	>20	<20	0	0	0	0
	693	C	C	AC	>20	C	C	AC	>20	C	C	AC	>20	0	0	0	0
H.influenzae	T281	not done				0	0	0	0	C	C	>20	>20 L	C	C	>20	>20 L

I = Reciprocal of fraction of Brown's opacity 1
L = Large colonies. Remaining growths gave medium-sized colonies.
. = No result - either not done or contamination has occurred.

The strains in Table 23 were chosen for the main growth factor experiments, because they were representative of the main clinical groups. T30, T145 and 693 were isolated from puerperal pyrexia cases, T46 from an ante-natal, T91 from a gynaecological and T94 from a post-menopausal case.

H. influenzae, which grew luxuriantly in all inoculum dilutions on autoclaved blood agar plus pure co-enzyme I, and on serum agar plus X and V, failed to grow on autoclaved blood agar itself, whereas HILB strains grew as well on this as on blood agar. This shows that V factor (and also incidentally catalase, which is destroyed by autoclaving) is not required by HILB.

In Tables 24, 25 and 27, the failure of X and V factors to promote growth of HILB on ordinary media, solid or fluid, is seen. Table 28 shows the same failure when serum agar is used, and a repetition of this experiment showed that the same failure occurred when haematin was used in a dilution of 1/1,000,000. The use of high dilutions of X is also seen in Table 26, where 4 different concentrations of X failed to give growth from a heavy inoculum in the majority of cases. The failure of charcoal to promote growth is shown in this table and also in Table 30.

Table 28 shows a similarity between H. canis

TABLE 24

X and V in digest agar.

	Growth after 72 hours incubation									
	Digest Agar + X + V					Blood Agar Control				
Inoculum Dilution	10	10^2	10^3	10^4		10	10^2	10^3	10^4	
Strain										
T 30	0	0	0	0		C	AC	>20	<20	M
T 46	0	0	0	0		C	AC	>20	>20	M
T 91	0	0	0	0		C	C	>20	>20	M
T 94	0	0	0	0		C	AC	>20	>20	M
T145	0	0	0	0		C	AC	>20	>20	M
693	0	0	0	0		C	C	AC	>20	L
H. canis	AC	0	0	0	±	C	C	AC	>20	L
H. influenzae T281	C	C	>20	>20	L	.	.	.	.	

X = haematin 1/33,000 V = Co I, 1/66,000

0 = no growth ± = very small colonies

M = medium-sized colonies L = large colonies

. = not done, or no reading obtained.

TABLE 25. Effect of X and V in ordinary agar

Growth after 72 hours incubation																				
	OA				OA + X				OA + V				OA + X + V				Blood Agar			
Inoculum[I] Dilution	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4
Strain																				
T 30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	C	AC	>20	>20
T 46	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	C	C	AC	>20 2
T 91	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	C	AC	>20	>20 2
T 94	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	C	AC	>20	<20 2
T281 H. influenzae	0	0	0	0	C	0	0	0 ±	C	0	0	0 ±	C	C	C	>20 L	C	C	tr	0 ±

OA = ordinary agar (2% peptone)
X = haematin, 1/33,000
V = Co. I, 1/66,000
I = reciprocal of fraction of Brown's opacity 1.
2 = 48 hours incubation
± = very small colonies
tr = trace
L = large colonies

TABLE 26

Haematin and charcoal in digest agar

	Growth at 48 hrs. 37°C.									
	H $\frac{1}{2 \times 10^6}$		H $\frac{1}{2 \times 10^5}$		H $\frac{1}{2 \times 10^4}$		H $\frac{1}{600}$		Blood Agar Control	Digest Agar Control
		+C		+C		+C		+C		
Strain										
693	-	-	-	-	-	-	-	-	++	-
584	-	-	-	-	-	-	-	-	+	-
623	-	-	-	-	+	-	-	-	++	-
239	-	-	-	-	-	-	-	-	+	-
400 (H.influenzae)	-	-	† +	-	† ++	-	.	.	+++	-

H = haematin

C = charcoal, 0.5%

. = not done

† = contaminant present

\+ = scanty growth

++ = moderate growth

+++ = heavy growth

\- = no growth

Table 27

X and V in fluid medium.

Failure to allow growth of HILB.

Strain	2% peptone broth + haematin 1/10,000	peptone broth + Co. I 1/50,000	peptone broth + haematin + Co. I.
877 H.influenzae	-	-	+
693	-	-	-
966	-	-	-
976	-	-	-

+ and - indicate whether growth had taken place after 3 days aerobic incubation at 37°C.

Co. I = co-enzyme I.

TABLE 28.

X and V factors in serum digest agar, and a comparison of HILB and H. canis.

Strain	Growth after 72 hours. T94				T145				693				H.canis*			
Inoculum dilution	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4
Medium.																
FSD	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FSD + X	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Blood agar	C	AC	>20	>20 M	C	AC	>20	>20 M	C	C	AC	>20 L	C	C	AC	>20 L

FSD = Filtered serum digest agar.

X = haematin 1/33,000

* = National Collection of Type Cultures. no. 8540.

Other abbreviations as in similar tables elsewhere.

This experiment was also done with X in a concentration of 1/1,000,000, with exactly the same results.

and HILB, neither being able to grow on serum digest agar plus X, although H. canis is supposed only to require X factor for growth.

B. Effect of haemoglobin and its derivatives, other than haematin.

The haemoglobin concentration in blood is 14%. The amount of haemoglobin to be added to give a similar concentration to that in 5% blood agar is therefore 14% of 5%. i.e. 0.7%. In fact, owing to difficulty in dissolving and Seitz-filtering the pure haemoglobin which was obtained, the maximum concentration obtainable was a 2.5% solution, of which 30 ml. was added to 100 ml. digest agar, giving a concentration of approximately 0.5%.

Haem and haemin occupy approximately 5% of the haemoglobin molecule. Accordingly, to keep the proportions similar to those in blood agar, 5% of 0.7% was used, i.e. about 1/3000, For details of solutions, see Appendix nos. 49 and 50.

Heavy inocula of HILB were used for this experiment, and 5 days incubation at 37°C. (aerobic). The results are seen in Table 29.

No growth occurred on any of these media. The routine subcultures on blood agar served as controls on the viability of the inocula.

The growth factor present in stromata and some batches of plasma is not therefore haemoglobin,

TABLE 29

Haemoglobin, haemin and haem in digest agar

	Haemoglobin Digest Agar	Haemin Digest Agar	Haem Digest Agar
Strain 693	-	-	-
584	-	-	-
623	-	-	-
880	-	-	-
239	-	-	-

haemin or haem.

C. <u>Effect of catalase and comparison with H. pertussis</u>

In view of work by Mazloum and Rowley (1955) on the effect of catalase and catalase-containing extracts of staphylococci the following tests were done to compare HILB with H. pertussis (phase I) in this respect. Cohen and Wheeler's medium, as modified by Mazloum and Rowley, was used (see appendix no. 54) with and without catalase. The effect of catalase in thioglycollate medium was also investigated. The results are seen in Tables 30 - 32.

Only very scanty growth from large inocula occurred on C.W. + charcoal + catalase with HILB, whereas H. pertussis grew luxuriantly on the same medium. H. canis obviously required more than X factor (whose function catalase should bypass) as growth was poor on the catalase C.W. However, growth did occur, even in high dilutions, the number of colonies being the same as in blood agar. The growth of pertussis on C.W. itself was fairly good but required a heavier inoculum than on C.W.C. plus catalase. No growth of HILB occurred on C.W. without catalase.

The effect of adding catalase to serum digest agar is seen in Table 31. Catalase made no improvement in the 5% filtered serum agar(which was

TABLE 30.

Cohen and Wheeler's medium without without catalase

	Growth after 72 hours incubation															
	Cohen and Wheeler agar (CW)				Cohen and Wheeler + catalase				Cohen and Wheeler + charcoal + catalase(CWC)				Blood agar control			
Inoculum[I] Dilution	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4
Strain																
T 30	0	0	0	0	0	0	0	0	0	0	0	0	C	AC	>20	<20
T 46	0	0	0	0	0	0	0	0	C±	0	0	0±	C	AC	>20	>20
T 91	0	0	0	0	0	0	0	0	0	0	0	0	C	C	>20	>20
T 94	0	0	0	0	0	0	0	0	C±	0	0	0	C	AC	>20	>20
T145	0	0	0	0	0	0	0	0	C±	0	0	0	C	AC	>20	>20
693	0	0	0	0	0	C	0	0	C	C	0	0	C	C	AC	>20 L
H. canis	0	0	0	0	0	C	AC	>20±	C	C	AC	>20±	C	C	AC	>20 L
H. pertussis	C	AC	>20	0	contaminated				C	C	AC	>20L	C[1]	C	>20	>20 ±

I = reciprocal of fraction of Brown's opacity

L = large colonies

± = very small colonies

remainder = medium sized

1 = 48 hours incubation

TABLE 31

Test using 5% filtered serum digest agar with and without catalase:

72 hours growth															
	5% filtered serum digest agar					5% filtered serum digest agar + catalase					Blood agar control				
Inoculum[I] Dilution	10	10^2	10^3	10^4		10	10^2	10^3	10^4		10	10^2	10^3	10^4	
Strain															
T 30	0	0	0	0		0	0	0	0		C	AC	>20	>20	M
T 46	AC	0	0	0	±	AC	0	0	0	±	C	AC	>20	>20	M
T 91	C	0	0	0	±	0	0	0	0		C	AC	>20	>20	M
T 94	C	>20	0	0	†	AC	<20	0	0		C	C	AC	>20	M
H. canis	AC	>20	<20	0	±	C	AC	>20	>20	L	C	C	>20	>20	L
T281 H. influenzae	C	AC	>20	0	±	C	AC	>20	>20	M	C	C	>20	>20	†

I = Reciprocal of fraction of Brown's opacity

L = large colonies

M = medium sized colonies

† = small colonies

± = very small colonies

TABLE 32.

Thioglycollate broth* with and without catalase.

	T238 + catalase		T238 (no catalase)	Control Tube + catalase
	$\frac{1}{10}$	$\frac{1}{1000}$	$\frac{1}{100}$	
Anaerobic Growth 4 days	+++	+++	+++	-
Catalase Activity after 4 days	+	+	-	++

+ = moderate number of bubbles.

++ = numerous bubbles.

- = no bubbles, or no growth.

* with 5% plasma.

on the borderline of growth-promotion as far as HILB were concerned). The difference in growth between filtered serum agar and blood agar could therefore not be accounted for by the lack of catalase in the former. In contrast, H. canis showed a marked improvement in growth when catalase was added, both in colony numbers and size, growing in fact as well on filtered serum plus catalase agar as it did on blood agar.

T 238, when grown in plasma thioglycollate broth, showed a persistently anaerobic distribution of growth. It was thought that an inadequate supply of catalase in the medium might cause this and so the following experiment was carried out:-

One ml. Oxoid sterile catalase solution in phosphate buffer, which had been freeze-dried and stored for several months in the refrigerator, was reconstituted. (One ml. of the original solution was stated to decompose 35 g. H_2O_2 in 10 minutes at 25°C.) Two drops of the reconstituted solution were added to 0.5 ml. volumes of Brown's opacity 1/10 and 1/1,000 suspensions in digest broth of a 48-hour blood agar culture of T 238. The mixture was tested for catalase activity and gave vigorous effervescence with 10-volume H_2O_2. Approximately 0.2 ml. of the emulsion was then inoculated into each tube of medium with a Pasteur pipette in the

same manner as previously. After 4 days incubation, growth was as shown in Table 32. A control tube was "inoculated" with a corresponding amount of catalase solution but no organisms.

After 4 days incubation all three inoculated tubes showed the same heavy growth with definite anaerobic distribution. To test for remaining catalase two ml. 10-volume H_2O_2 was poured carefully on to the medium and the results were as shown in the table. The catalase had remained active throughout the incubation period but had had no effect in changing the anaerobic distribution of growth. Note that the amount of catalase in the plasma was not sufficient to give a positive test.

D. <u>The effect of different species of blood</u>

This was investigated by method c. The media were made up as described in the Appendix (no. 4), sterile citrated blood of horse, ox, sheep and rabbit being substituted for human blood.

The results are seen in Table 33.

As far as the number of colonies is concerned it will be seen from the table that these were comparable for all species of blood, but the size of the colonies showed some variation.

The four HILB strains all produced "medium-sized" colonies on human blood agar after 3 days incubation, i.e. about 1/3 mm. in diameter. On

TABLE 33. Effect of different blood species

	Growth after 72 hours incubation																								
Species of blood used	Human					Horse					Ox					Sheep					Rabbit				
Inoculum Dilution	10	10^2	10^3	10^4		10	10^2	10^3	10^4		10	10^2	10^3	10^4		10	10^2	10^3	10^4		10	10^2	10^3	10^4	
Strain																									
T 30	C	AC	>20	>20	M	C	AC	>20	>20	M	C	AC	>20	>20	ǂ/v	C	AC	>20	>20	ǂ/v	C	AC	>20	>20	ǂ/v
T 46	C	AC	>20	>20	M	C	AC	>20	>20	M	C	AC	>20	>20	ǂ/v	C	AC	>20	>20	ǂ	C	AC	>20	>20	ǂ
T 91	C	AC	>20	>20	M	C	AC	>20	>20	M/v	C	AC	>20	>20	ǂ	C	AC	>20	>20	M	C	AC	>20	>20	ǂ
T 94	C	C	AC	>20	M	C	>20	>20	>20	ǂ/v	C	AC	>20	>20	ǂ/v	C	AC	>20	>20	M/v	C	AC	>20	>20	ǂ
T281 (H. influenzae)	C	C	>20	>20	ǂ	C	AC	>20	>20	ǂ	C	AC	>20	>20	±	C	C	>20	>20	±	C	C	C	>20	L
H. canis	C	C	>20	>20	L	C	AC	>20	>20	L	C	AC	>20	>20	L	C	C	>20	>20	L	C	C	AC	>20	L

Abbreviations as in other similar tables.

horse blood agar T 30 and T 46 gave similar-sized colonies but T 91 showed variability in size (a sign of a poor medium) and T 94 gave small colonies, also variable in size. On ox and rabbit blood agar, all four strains gave small colonies, with some variability, and on sheep blood T 30 and T 46 gave small colonies, while T 91 and T 94 gave medium-sized colonies, variable in the case of the latter.

A marked contrast is seen with H. influenzae (T 281) which gave small colonies on human and horse, very small (barely visible) on ox and sheep, but large and luxuriant on rabbit blood agar.

H. canis also showed a marked difference from both HILB and H. influenzae in that the colonies in all five species of blood agar were equally large.

E. Investigation into a batch of defective blood.

It was noticed on certain occasions that a particular batch of blood failed to yield satisfactory growth. Blood agar plates are always marked according to the type of peptone, batch of blood and batch of plates, e.g. E12 meant that Evans' peptone had been used, number 1 blood bottle was used, and the plate was from the second batch of pourings.

It was found that batch number E61 gave very poor growth, although a duplicate plate of batch

E55 which had been inoculated at the same time, gave good growth. It was decided to try to trace the cause of this failure to support growth. There seemed no difference from a normal batch, except for the failure of HILB to grow on it, as Strep. pyogenes, H. influenzae and pneumococci were all growing normally on it, during routine work. This was thought to rule out any antibiotic or sulphonamide content of the blood, due to treatment of the donor. It was decided to try adding to the surface of the medium (no more blood of that batch was available to make further plates) various growth factors and anti-inhibitors.

First the effect of V factor from Staph. aureus was tried. E61 was inoculated with 7 HILB strains, and a single streak of 24-hour Staph. aureus culture was inoculated across each strain. After 24 hours incubation at 37°C. aerobically, results were as in Table 34.

Table 34. Growth on defective blood agar. Effect of neighbouring staphylococci:

Strain	Growth away from Staph.	Growth next Staph.
T34	-	+
T35NH	-	+
T38	++	++
T46	+++	++
T60	+	+
T62	+++	+++
T98	-	++

+ = scanty growth
++ = moderate growth
+++ = heavy growth

It was noted that certain strains could grow on the medium but that others required the presence of staphylococcus culture for growth. As V factor satellitism had never before been noted, this was puzzling until it was realised that the staphylococcus used was a naturally occurring penicillin-resistant strain and was therefore a producer of penicillinase. It was then decided to try penicillinase as well as other factors as seen in the next table (35). As inoculum, a faintly turbid suspension in digest broth of a 48-hour blood agar culture was used (1 drop).

Co-enzyme I was used in 1/1000 solution (Seitz-filtered), 1 drop being added to the plate beside each inoculated area. Haematin 1/10,000 autoclaved solution, digest, yeast extract, batch 7 blood, 5% starch, 3% mucin and penicillinase were added in a similar manner. As control, an E7 batch of blood agar was used.

It can be seen from table 35 that penicillinase was the only substance having any definite effect. Could the penicillinase preparation also contain some growth factor preserved during manufacture? To test this, it was decided to try a penicillin-sensitive staphylococcus to see if it had the same growth-promoting action of the penicillin-resistant strain already used. The

TABLE 35.

Addition of various substances to defective blood agar.

Strain	Alone	+ CO I	+ Haematin	+ Digest	+ yeast Extract	+ 7 blood	+ starch	+ mucin	+ Penicill-inase	E 7
T34	±	-	-	±	-	±	±	-	++	++
35NH	±	-	-	±	±	±	±	-	++	++
46	±	++	+	++	++	+++	++	++	++	++
98	-	-	-	-	-	±	±	-	++	++
100	-	-	-	-	-	±	-	-	++	++
104	±	-	-	-	-	±	±	-	++	++
109	-	±	±	+	+	±	+	±	++	++

Co.I = Co-enzyme I.

E 7 = batch of blood agar made with blood 7.

± = doubtful growth, barely visible.

+ = scanty growth.

++ = moderate growth.

+++ = heavy growth.

previous experiment was therefore repeated using the Oxford standard staphylococcus (NCTC 6571). Poor growth of the staphylococcus occurred, and round the colonies which did develop, no satellitism occurred, thus proving that the growth-promoting action of the previous staphylococcus must have been due to its penicillinase production. Later on, other batches of blood with similar, but not so marked, action were encountered and cured in the same way by adding penicillinase in very small quantity. The amount actually used is given in the Appendix (No. 4). This amount was so small that it had no appreciable effect on the size of zones of inhibition of penicillin disks used in routine diagnostic work, but it was sufficient to deal with the traces present in the medium. As a precautionary measure, penicillinase is now added as a routine to all blood agars produced.

F. <u>Effect of yeast extract</u>:

1. <u>In blood agar</u>:

5% yeast extract, prepared as in Appendix no. 52, was added to the routine 6% human blood 5% digest agar. Ampoules of freeze-dried cultures were opened, reconstituted in broth and the inoculum divided equally between ordinary blood agar (BA) and blood agar plus yeast extract (BAY). The results were read after 48 hours aerobic incubation

at 37°C., and are seen in Table 36. Sub. 1 and 2 refer to sub-cultures carried out successively at 48-hour intervals. The experiment was then repeated with 0.5% yeast extract instead of 5% and using established cultures instead of dried ones. One sub-culture was made after 4 days. The results are given in Table 37.

There is obviously little to choose between BA and BAY in Table 36, but in Table 37 it can be seen that an improvement in the sub-culture after 4 days was caused by the addition of 0.5% yeast extract.

2. <u>In blood agar broth (BAB)</u>:

Details of this medium are given in the Appendix (No. 20). It consists of plasma digest broth superimposed on blood agar in a tube. This medium was tested with and without the addition of 5% yeast extract. (BAB and BABY). Results are given in Table 38. The readings were made after 48 hours aerobic 37°C. incubation, and sub-cultures were made at the same time. It can be seen from the table that little if any improvement was caused by the addition of yeast extract.

G. <u>Use of washed red cells instead of blood</u>:

Human red cells were washed four times in normal saline and made up with saline to the original blood volume. Six per cent. of this suspension

TABLE 36.

Growth on blood agar with and without yeast extract, of freeze-dried cultures.

Strain	BA	BAY	Sub. 1		Sub. 2	
			BA	BAY	BA	BAY
T 7	-	+	++	+++	+++	+++
10	++	+++	++	+++	++	+
12	+++	+++	++	c	+++	+++
15	+++	++	++	+++	+++	+++
30	++	++	++	+++	+++	+++
33	+++	+++	+++	+++	+++	+++

c = contaminated. Growth symbols as in Table 35.

TABLE 37.

Growth on blood agar with and without yeast extract.

Strain	BA	BAY	Sub. after 4 days	
			BA	BAY
T 7	+++	++	+	+++
10	++	++	+	++
12	++	++	+	±
15	+++	++	+	++
30	++	++	-	++
33	+++	+++	+	++

Growth symbols as in Table 35.

TABLE 38.

Growth in blood agar broth, with and without yeast extract.

	BAB		BABY	
Strain	Apparent growth	Sub-culture on BA	Apparent growth	Sub-culture on BAY
T 7	++	+++	++	+++
10	+	+++	++	++
12	c	c	++	++
15	c	c	++	+++
30	++	+++	++	+++
33	+++	+++	+++	+++

c = contaminated

Other symbols as in Table 35.

was then added to 5% digest peptone agar. This was called RCDA. Comparison was made with digest agar plus 5% plasma obtained from the same blood as the red cells (PDA) and with ordinary blood agar (BA). Inoculation was direct, with a loop, from 48-hour blood agar cultures. Incubation was for 3 days aerobically at 37°C. and the first sub-cultures were made at the end of that time. The second sub-cultures were made after a further 48 hours. The results are seen in Table 39.

Growth can be seen to be nearly as good on red cell digest agar as on blood agar, and this was maintained through 2 successive sub-cultures.

H. Effect of red cell stromata: and their main constituents:

i. Stromata:

Human red cell stromata were prepared by the method given in Starling's Principles of Human Physiology (Lovatt Evans, 1947) page 486. After repeated washing with sterile distilled water, until the washing water was colourless, 3 ml. of the packed stromata were added to 150 ml. digest agar and plates poured. Table 40 shows the results of 2 trials of the medium. For negative control of the 1st. trial, 0.0003% haemoglobin digest agar was inoculated at the same time as the test medium. Incubation periods were 48 hours (aerobic) at 37°C. unless otherwise specified.

TABLE 39.

Substitution of washed red cells for blood.

	Growth after 3 days at 37°C.								
	RCDA			PDA			BA		
HILB Strain		Sub.1	Sub.2		Sub.1	Sub.2		Sub.1	Sub.2
351	++	+	++	+++	++	+++	+++	+++	+++
349	++	++	+++	+++	++	++	+++	+	+++
548	+++	+++	++	+	+++	+++	+++	++	+++
968	±	±	+	++	+	++	+	+	++
629	±	±	+	++	+	++	++	++	++
693	+++	++	++	+++	+++	+++	+++	+++	+++
H. influenzae Strains									
877	+++	+++	+++	±	-		+++	+++	+++
882	++	+++	+++	±	-		++	++	+++

+ = scanty growth

++ = medium growth

+++ = heavy growth

- = no growth

± = doubtful growth

TABLE 40.

Growth on red cell stroma digest agar.

Strain	Experiment 1		Experiment 2		
	2% stroma digest agar	haemo-globin digest agar	2% stroma digest agar (1)	Sub-cult. from (1) on stroma digest agar (2)	Sub-cult. from (2) after 4 days: on blood agar
693	+++	-	++	+++	++
623	+++	-	++	+++	++
281	++	-	•	•	•
239	+++	-	+++	+++	++
880	•	•	+++	+++	+

+ = scanty growth

++ = moderate growth

+++ = heavy growth

• = no result

In the first experiment moderate or heavy growth occurred with 4 strains, the possible effect of carry-over of growth factors from the heavy inoculum being minimised by the lack of growth in the control. In the 2nd. experiment, good growth was maintained in sub-culture and remained viable for at least 4 days.

Effect of boiling stroma digest agar:

This time the stromata were washed 3 times and were reconstituted to original blood volume with sterile distilled water. Two per cent. of this suspension was added to digest agar along with 0.25% glucose and 0.005% bromthymol blue. The object was to test the suitability of stroma agar as a base for sugar fermentation tests. The boiling was momentary, as for Levinthal's agar. Results were read after 3 days aerobic incubation at 37°C. The results are seen in Table 41.

Table 41. Boiled stroma digest agar

Strain	Boiled Stroma Agar	Unboiled Stroma Agar
693	+++	+++
584	-	-
623	+++	+++
880	++	++
239	-	-

+++ = heavy growth ++ = moderate growth

- = no growth

The routine blood agar sub-cultures provided a control of the viability of the inocula. Carry-over could have been a factor in this experiment, but carry-over of growth factor has never been able to produce good growth of HILB, in our later experience of the use of serially diluted inocula. Boiled stromata therefore seem to support growth as well as unboiled.

ii. Stroma constituents:

According to Lovatt Evans (1947) red cell constituents are as follows:-

37% of red cells are solids and 0.3% of these solids are stroma lipides. 60% of these are phospholipides (half of which is cephalin), 30% is free cholesterol and 10% are cholesteryl esters and fats. As 5% of undiluted blood is used in blood agar, the proportion of red cells in the medium is approximately half that, i.e. say 2%. The proportion of red cell phospholipide in the medium is therefore 60% of 0.3% of 37% of 2%. i.e. roughly 1/100,000. The proportion of cephalin and cholesterol is therefore 1/200,000 (approximately).

The proportion of stroma protein in red cell solids is 4%. The proportion in blood agar is therefore 4% of 37% of 50% of 5%, i.e. approximately 0.04%. Nucleic acid forms roughly half the stroma protein, so that the proportion of nucleic

acid in blood agar is about 0.02%.

It was decided to use cholesterol and cephalin at double the above proportions, i.e. 1/100,000, and nucleic acid at 0.02%, or 1/5000. To represent the remaining phospholipides, lecithin was used in a concentration of 1/100,000. These substances were prepared and added to digest agar as described in the Appendix (nos. 65 - 69). It was hoped that these constituents of stromata might, at least partially, replace red cells in culture media. The above media were therefore tested by inoculating them with strains T 30, T 91, T 94 and T 145 by method c, in duplicate, half the plates being incubated aerobically, the other half anaerobically. Strains T 143 and T 98 were also inoculated at the same time, on aerobic plates only. These were peroxidase positive strains, which it was hoped might be able to grow aerobically under conditions where the other peroxidase negative strains could only grow anaerobically. The results are seen in Table 42.

It will be seen that digest agar gave poor (anaerobic) growth, colonies being very small. A preliminary experiment had shown that digest agar failed to support growth in sub-culture, while cholesterol digest agar continued to support growth for 3 successive cultures of 4 strains and at least 4

TABLE 42. Cholesterol, lecithin, cephalin and nucleic acid in digest agar.

Effect of X factor

	Growth after 72 hours																							
	T 30				T 91				T 94				T145				T143				T 98			
Inoculum Dilution	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4
Medium (1) Aerobic																								
DA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ChDA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ChDA+X	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LDA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CDA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
NDA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LCChNDA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LCChNDA+X	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BA	C	C	AC	>20M	C	C	>20	>20M	C	AC	>20	>20M	C	AC	>20	>20M	C	AC	>20	>20ɫ	AC	>20	<20	0 M
(2) Anaerobic																								
DA	C	>20	>20	0±	C	>20	>20	>20ɫ	AC	AC	<20	<20±	C	AC	>20	<20±								
ChDA	C	AC	>20	<20ɫ	C	AC	>20	<20MV	C	AC	>20	<20ɫ	C	AC	>20	>20ɫ								
ChDA+X	0	0	0	0	0	0	0	0	0	0	0	0	AC	0	0	0 ±								
LDA	.	AC	>20	<20L	C	AC	>20	<20L	AC	AC	>20	<20MV	C	AC	>20	>20MV								
CDA	C	AC	>20	<20MV	C	AC	>20	<20MV	AC	AC	>20	<20ɫV	AC	AC	>20	<20ɫ								
NDA*	C	AC	>20	<20M	C	AC	>20	<20MV	C	AC	>20	<20ɫV	C	C	>20	<20ɫ								
LCChNDA	AC	>20	<20	0 ɫV	C	>20	>20	<20MV	AC	>20	>20	<20ɫV	C	AC	.	<20M								
LCChNDA+X	0	0	0	0	>20	<20	0	0 ±	>20	0	0	0 ±	C	AC	>20	<20±								

DA = digest agar (Appendix No.7).
ChDA = digest agar + cholesterol (Appendix No.65)
ChDA+X = digest agar + cholesterol + X $\frac{1}{33,000}$
LDA = digest agar + lecithin (Appendix No.66)
CDA = digest agar + cephalin (Appendix No.67)
NDA = digest agar + nucleic acid*
LCChNDA = digest agar + all 4 above.
BA = blood agar.

Abbreviations as in other tables, except ɫ = small colonies

* nucleic acid inadvertently used in 1/50,000 concentration instead of 1/5000 as intended, but later work showed no improvement on using the higher concentration.

successive cultures of 2 strains, starting in all cases with dilute inocula. The strains used were the first four in Table 42.

Cholesterol, lecithin, cephalin and nucleic acid all gave definitely improved growth compared with digest agar alone, but growth only occurred under anaerobic conditions. This suggested requirement of X factor, but when X factor was present, inhibition of growth occurred anaerobically, and aerobic culture was negative also. The 2 peroxidase positive strains T 143 and T 98 also failed to grow aerobically, except on the control blood agar.

The result of combining the above substances in one medium is shown in the table also. LCChNDA was no better than any of the other media, indeed on the whole it was not quite so good. The addition of X factor to this last was not quite so disastrous as to ChDA, but was obviously causing inhibition of growth.

The X-containing media were controlled by growing an H. influenzae strain on ChDA, with and without X, adding 1 loopful of 1/1000 co-enzyme I in each case. Heavy growth occurred on the X-containing medium, but none on the control ChDA +V.

I. Effect of extracts of blood in culture media:

1. Fildes' peptic digest of blood (Fildes, 1920)

Fildes' peptic digest of blood was heated half

an hour at 56°C. to remove the chloroform, and added to ordinary agar in 5% concentration before pouring plates. Four HILB strains were tested for growth by method "c", and the results are seen in Table 43.

No growth, either of heavy or dilute inocula, occurred. Anaerobic incubation gave the same negative result as aerobic. A strain of H. influenzae isolated from sputum, however, gave luxuriant growth after 24 hours aerobic 37°C. incubation, when plated out from a diluted inoculum.

2. Red cell extract:

Washed human red cells were boiled in broth, centrifuged and the supernatant added to digest agar in 10% concentration. A similar extract was made by autoclaving red cells in broth at 120°C. for half an hour. This was also used in 10% concentration in digest agar. Using method "c" the following results were obtained (v. Table 44).

None of the following HILB strains could grow in any dilutions:- T 30, T 46, T 91, T 94, T 145, 693, although the control blood agar showed growth in all 4 dilutions. T 281 (H. influenzae) grew poorly, in the first 2 dilutions, on boiled red cell extract, but not on autoclaved extract.

3. Lysed blood:

Lysed blood agar plates as used for sulphonamide

TABLE 43.

Fildes peptic digest of blood agar.

		Growth after 72 hours at 37°C.												
		FPDBA aerobic				FPDBA anaerobic				Blood agar aerobic				
Inoculum dilution		10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4	
	T 30	0	0	0	0	0	0	0	0	C	AC	>20	<20	M
HILB	T 91	0	0	0	0	0	0	0	0	C	>20	<20	<20	M
strain	T 94	0	0	0	0	0	0	0	0	C	AC	>20	<20	M
	T145	0	0	0	0	0	0	0	0	C	AC	>20	<20	M

FPDBA = Fildes peptic digest of blood agar.

Abbreviations otherwise are as in other similar tables.

TABLE 44

Red cell extracts.

	Growth after 72 hours incubation											
	Boiled red cell extract				Autoclaved red cell extract				Blood Agar control			
Inoculum Dilution	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4
Strain												
T 30	0	0	0	0	0	0	0	0	C	C	AC	>20
T 46	0	0	0	0	0	0	0	0	C	C	AC	>20
T 91	0	0	0	0	0	0	0	0	C	AC	>20	>20
T 94	0	0	0	0	0	0	0	0	C	AC	>20	>20
T145	0	0	0	0	0	0	0	0	C	AC	>20	.
693	0	0	0	0	0	0	0	0	.	AC	>20	>20
T281 H. influenzae	C	AC	0	0 ±	0	0	0	0	C	AC	0	0 ±

Abbreviations as in other similar tables.

TABLE 45.

Lysed blood agar.

Inoculum Dilution	Growth after 72 hours incubation									
	Lysed blood agar					Blood agar control				
	10	10^2	10^3	10^4		10	10^2	10^3	10^4	
Strain T 30	0	0	0	0		C	AC	>20	<20	
T 46	•	•	0	0		C	AC	>20	<20	
T 91	0	0	0	0		C	AC	>20	<20	
T 94	0	0	0	0		C	AC	>20	<20	
T145	AC	>20	0	0	±	C	AC	>20	<20	
693	AC	>20	0	0	±	•	AC	>20	>20	
T281										
H.influenzae	C	AC	>20	>20	±	C	AC	>20	>20'	
H.canis	C	C	>20	<20	L	C	AC	>20	<20'	L

' = 48 hours incubation only.
Other abbreviations as in previous tables.

sensitivity testing (Harper and Cawston, 1945, modified) were tried. For details of the medium, see Appendix (no. 3). This medium contained 10% of lysed horse blood. The results are seen in Table 45. Three of the 6 strains used failed to grow even in heavy inoculum. Two strains, T 145 and 693, grew poorly in the first two dilutions and the remaining strain failed to grow in the last two dilutions (dilutions 1 and 2 were contaminated). All control plates grew in all dilutions. H. influenzae grew in all dilutions, but with very small colonies, as on blood agar. H. canis gave its usual large colonies on this medium, showing once again the difference in growth requirements from HILB.

J. Determination of the best combination for fluid medium.

The constituents to be tested were plasma, digest, maltose and Evans peptone broth. Method "a" was used, 3 successive cultures in the same medium being carried out, with a final sub-culture on to blood agar in order to assess whether growth had been continued. This was necessary because of the difficulty of assessing growth in fluid medium containing plasma. The following combinations of constituents were made up:-

B = 2% Evans peptone broth (Appendix no. 1)

DB = 2% Evans peptone broth + 5% digest (Appendix no. 32)

MDB = 2% Evans peptone broth + 5% digest + 0.25% maltose (v. Appendix 37)

PB = 2% Evans peptone broth + 5% human plasma.

PDB = 2% Evans peptone broth + 5% human plasma + 5% digest (Appendix 35)

PMDB = 2% Evans peptone broth + 5% human plasma + 5% digest + 0.25% maltose

PMB = 2% Evans peptone broth + 5% human plasma + 0.25% maltose

D = 50% digest broth (Appendix no. 6)

PD = 50% digest broth + 5% plasma

One drop 24-hour culture in PMDB (37°C. aerobic) was added to each 3 ml. tube of medium to be tested, except in the case of strain 839, where growth was poor and 4 drops were used as inoculum. After 48 hours 37°C. aerobic incubation, 4 drops from each tube were added to a second tube of the same medium and these were again incubated 48 hours at 37°C. Sub-culture was then repeated in the same medium and finally, after 48 hours incubation of the 3rd. successive culture, blood agar plates were inoculated with a loopful of the culture. Only the results of this final sub-culture on blood agar are given. The results are seen in Table 46.

B, DB, MDB, and D all failed completely to allow continued growth. Growth of 1 strain failed in PD and of 3 strains in PMB. The remaining 3 media, PB, PDB and PMDB all gave equally good growth. The common factor in all media giving

TABLE 46.

Different combinations of broth, digest, maltose and plasma.

Medium	Final Blood Agar subculture after 3 successive cultures					
	Strain					
	693	623	839	880	239	837
B	-	-	-	-	-	c
DB	-	-	-	-	-	-
MDB	-	-	-	-	-	-
PB	+++	+++	+++	+++	+++	++
PDB	+++	+++	+++	+++	+++	+++
PMDB	+++	+++	+++	++	+++	+++
PMB	+	-	+++	+++	-	-
PD	+++	+++	-	+++	+++	++
D	-	-	-	-	-	-

+ = scanty growth

++ = moderate growth

+++ = heavy growth

- = no growth

growth was plasma.

K. Serum compared with plasma in fluid medium. Effect of Seitz-filtering.

Five per cent. Seitz-filtered human serum was added to digest broth (FSDB) and the medium compared with 5% plasma digest broth (PDB). The media were inoculated from 48-hour cultures of HILB, and sub-cultured on to blood agar after 48 hours incubation. Table 47 shows the growth results in blood agar sub-culture.

Table 47. Growth in plasma and filtered serum broth compared.

Strain	Blood agar sub-culture from	
	PDB	FSDB
693	+++	-
839	+	+
880	+++	-
837	++	++

It was thought that the Seitz-filtering of the serum might have reduced its efficacy. Serum was therefore tried unfiltered, made from 2 different batches of plasma (S_2 and S_3), and compared with PDB. Inoculation was as before, but the tubes were sub-cultured into further tubes of the

same medium and this sub-culturing repeated once again (3 successive cultures on SDB and PDB) before the final blood agar sub-culture was carried out.

The results are seen in Table 48.

Table 48. Growth in unfiltered serum broth compared with plasma broth

	Final blood agar sub-culture from:-				
Strain	S_2DB	S_3DB	S_2GDB*	S_3GDB*	PDB
693	c	++	++	++	+++
623	+++	++	c	+++	+++
839	+++	++	++	++	++
880	+++	+++	+++	+++	+++
239	+++	+++	++	+++	+++
837	+++	+++	c	++	+++

* = contains added glucose 0.25%
++ = moderate growth
+++ = heavy growth
c = contaminated

Serum in this case had just as good an effect as plasma. This may have been due to its being unfiltered, (contrast previous result with filtered serum) but the result is not conclusive, since the serum was obtained from a different source.

Ten per cent. filtered serum was later tried in digest broth with a few strains, to see if easily visible growth and good fermentation reactions could be obtained. One per cent. and 0.25% xylose

were added, and the tubes inoculated with a PDB suspension of 48 hours blood agar culture. Results were read after 4 days aerobic incubation at 37°C. and are given in Table 49.

Table 49. Growth and fermentation in 10% filtered serum broth

Strain	10% FSDB + 0.0025% bromthymol blue	10% FSDB + indicator + xylose:-			
		0.25%		1.0%	
	Visible Growth	VG	F	VG	F
T 38	-	+++	+++	+++	+++
T 217H	+++	±	±	-	-
T 216	+++	++	+++	-	-
T 183	+++	+	-	±	±
T 182	.	+++	±	+++	++

VG = visible growth
F = fermentation (blue → yellow)
+++ = marked
++ = moderate
+ = scanty
± = doubtful
- = negative
. = no result

The visible growth appeared to be superior to that obtained in 5% plasma digest broth and fermentation results fitted in with those already obtained for these strains using PDB as the basis for sugars. It was not considered worthwhile, however, to switch over to the use of 10% serum on a

TABLE 50.

Filtered serum agar versus unfiltered.

		Growth after 48 and 72 hours									
		Amies and Jones (filtered serum)					Amies and Jones (unfiltered serum)				
Inoculum dilution		10	10^2	10^3	10^4		10	10^2	10^3	10^4	
Strain T 30	48 hrs.	C	C	>20	>20	†	C	AC	>20	>20	M
	72 hrs.	C	C	>20	>20	V	C	AC	>20	>20	M
T 46	48 hrs.	C	C	>20	>20	†	C	AC	>20	>20	M
	72 hrs.	C	C	>20	>20	V	C	AC	>20	>20	M
T 91	48 hrs.	C	AC	>20	>20	†	C	AC	>20	>20	M
	72 hrs.	C	AC	>20	>20	V	C	AC	>20	>20	M

Abbreviations as in other similar tables.

Table 51.

Effect of twice filtered serum

	Growth after 48 hours									
	Amies and Jones (twice filtered serum)					Blood Agar Control				
Inoculum Dilution	10	10^2	10^3	10^4		10	10^2	10^3	10^4	
Strain T 91	0	0	0	0		C	AC	>20	>20	
T 94	0	0	0	0		C	AC	>20	>20	
T281 H.influenzae	C	C	C	>20	L	C	C	tr	0	±

tr = trace

Remaining abbreviations as in other similar tables.

large scale, because of the extra expenditure of blood involved.

Using method "c" with serial dilutions of inoculum, the results in Table 50 were obtained, using Amies and Jones medium as the base (v. Appendix, no. 45). Amies and Jones (filtered) showed smaller colonies, with the strains shown, than Amies and Jones (unfiltered) but the difference was not marked, the number of colonies being the same in each case.

Table 51 shows the result when Amies and Jones' medium was made with twice filtered serum. Two strains of HILB, T 91 and T 94, failed altogether to grow on this medium, although H. influenzae grew luxuriantly, and the 2 HILB strains grew well on the blood agar control.

Ten per cent. filtered serum, when supported by 5% digest, can, however, give heavy growth, as seen below, both with Amies and Jones medium and 2% Evans peptone agar plus 5% digest, (FSD agar).

L. Amies and Jones' medium, with various alterations:

Amies and Jones' medium (AJ) as made in this laboratory, comprises 1% Bacto casitone (replacing tryptose), 5% yeast extract, 1% Lab Lemco (replacing beef heart infusion), sodium chloride, and 10% unfiltered serum. The phenol red used in the

original was omitted, (v. Appendix no. 45).

The following combinations, additions and replacements of AJ medium were tried:-

1. AJ minus serum (S).
2. AJ minus yeast extract (YE) and S.
3. AJ minus S and Lab Lemco (LL).
4. AJ minus LL.
5. AJ minus YE.
6. AJ minus LL and YE.
7. AJ minus LL, YE and S.
8. AJ using autoclaved YE. (AJautoYE)
9. AJ using Seitz-filtered serum (FS).
10. AJ using FS plus 5% digest (D).

Method "c" of inoculation and reading was used. The results are given in Table 52. The striking point arising from these results is the exceedingly poor growth caused by the withdrawal of Lab Lemco alone, as compared with the withdrawal of both Lab Lemco and yeast extract. Yeast extract appears to be doing actual harm to the medium in the absence of Lab Lemco. Serum is, as expected, the essential part of the medium. The removal of yeast extract seemed to make no difference to the growth. Lab Lemco, on the other hand improved the growth, as seen when AJ - LL - YE is compared with AJ - YE, the colonies in the latter being larger.

TABLE 52. Variations in Amies and Jones' medium

	Growth after 72 hours																							
Strain	T 30				T 46				T 91				T 94				T145				693			
Inoculum Dilution	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4
Medium:																								
AJ	C	AC	>20	>20	C	AC	>20	>20	C	AC	>20	>20	C	AC	>20	>20V	C	AC	>20	>20V	C	AC	>20	>20 V
AJ-S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AJ-S-YE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AJ-S-LL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AJ-LL	C	0	0	0 ±	C	>20	0	0 ±	C	>20	0	0 ±	C	0	0	0 ±	C	>20	0	0 ±	AC	0	0	0 ±
AJ-YE	C	C	AC	>20	C	C	AC	>20	C	AC	>20	>20	C	AC	>20	<20	.	C	>20	<20	C	AC	>20	>20
AJ-LL-YE	C	AC	>20	>20†	C	AC	>20	>20†	C	AC	>20	>20†	C	AC	>20	>20†	C	AC	>20	>20±	C	0	0	0 ±
AJ-LL-YE-S	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AJ(FS)	C	C	>20†	>20V	C	C	>20	>20V†	C	AC	>20	>20V	C	AC	>20	>20V	C	AC	>20	>20V	C	AC	>20	>20V
AJ(FS)+D	C	C	>20	>20L	C	C	>20	>20L	C	AC	>20	>20	C	AC	>20	>20	C	AC	>20	>20V	C	AC	>20	<20
AJ autoYE	C	AC	>20	>20	C	AC	>20	>20	C	AC	>20	>20	C	AC	>20	>20	C	AC	>20	>20V	C	AC	>20	<20
Blood agar control	C	C	AC	>20	C	C	AC	>20	C	AC	>20	>20	C	AC	>20	>20	C	AC	>20	.	.	AC	>20	>20

Abbreviations as in other similar tables, or in text

The basic medium of AJ is only 1% peptone, and it was thought that the stimulating action of Lab Lemco might not be so apparent if a better background was provided. Accordingly, 2% Evans peptone agar was used as a basis for further combinations of FS, LL, YE, and D.

The basic medium was 2% Evans peptone agar (Appendix no. 2) known as ordinary agar or OA. The following media, based on this, were made up.

1. OA plus 10% FS.
2. OA plus 10% FS plus LL.
3. OA plus 10% FS plus 5% D.
4. OA plus 10% FS plus 5% D plus LL.
5. OA plus 10% FS plus YE.
6. OA plus D plus LL.

The results are seen in Table 53. The same apparently harmful effect of YE is seen again, OA plus FS giving better growth than OA plus FS plus YE. LL has a contradictory effect, being both stimulating and inhibitory, as seen by the variable size of the colonies on OA plus FS plus LL, some of which were bigger than the small colonies obtained with OA plus FS alone. Digest was by far the best addition, next to serum. LL added along with digest did not improve the result, but merely gave variability to the colony size.

On this occasion, and on the previous ones (see tables 49 and 50) 10% filtered serum gave

TABLE 53. Variations in constituents of serum agar.

	Growth after 72 hours																							
Strain	T 30				T 46				T 91				T 94				T145				693			
Inoculum Dilution	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4	10	10^2	10^3	10^4
Medium																								
OA+FS	C	AC	>20	>20	C	AC	>20	<20†	C	AC	>20	<20†	C	AC	>20	>20†	C	AC	>20	<20†	C	AC	>20	>20†
OA+FS+LL	C	AC	>20	<20V	C	AC	>20	<20V	C	AC	>20	<20V	C	AC	>20	>20V	AC	AC	>20	<20V	C	AC	>20	<20V
OA+FS+D	C	AC	>20	<20L	C	AC	>20	<20L	C	AC	>20	<20L	C	AC	>20	<20L	C	AC	>20	<20L	C	AC	>20	>20L
OA+FS+D+LL	C	AC	>20	<20V	C	AC	>20	<20L	C	AC	>20	<20VL	C	AC	>20	<20VL	C	>20	>20	<20VL	C	AC	>20	<20VL
OA+FS+YE	C	AC	>20	0 ±	C	AC	>20	0 ±	C	20	>20	<20 V	AC	20	>20	<20	AC	>20	<20	0	C	AC	>20	<20±
OA+D+LL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Blood agar control	C	AC	>20	<20M	C	AC	>20	<20M	C	AC	>20	<20M	C	AC	>20	>20M	C	AC	>20	<20M	.	AC	>20	<20 M

Abbreviations as in other similar tables, or in text.

excellent results, especially with 5% digest. Unfortunately, later attempts to use this medium did not meet with the same success, presumably due to defective batches of serum.

M. <u>Effect of age of plasma digest broth constituents</u>:

After some disappointing results with 5% plasma digest broth, it was decided to compare the results when old constituents were used, with those for recently prepared materials. The only constituents likely to deteriorate with age were thought to be plasma, digest and yeast extract, (which was being used at this time in 0.5% concentration).

The following were the constituents, with their respective ages:-

Digest - old, 4 months; new, 6 weeks. (OD and ND).

Yeast extract - old, 3 months; new, 6 days.

Plasma - old, 6 months; new, 2 weeks.

The following permutations of these constituents were prepared.

a. Using Old Digest (OD)

1. Old yeast extract and old plasma.
2. Old yeast extract and new plasma.
3. New yeast extract and old plasma.
4. New yeast extract and new plasma.

b. Using New Digest (ND)

Same as above, 1, 2, 3, 4.

Each medium was made up with 0.25% of one of seven sugars, and the number of tubes being fermented by 4 HILB strains recorded. The medium giving the greatest number of positive results was considered the best and vice versa.

The method of inoculation was method (1), Chapter V, Section 1, 6 drops of 48-hour blood agar broth culture being added to 3 ml. of medium. Readings were made on the 5th and 14th days of incubation. Results are given in Table 54. It will be seen that some combinations of constituents gave more numerous positive results on both readings than others. Taking all 14-day positive tubes of all 4 strains as 1 each, and doubtful (±) as ½, the following figures are obtained:-

OD 1 +ve.	9	ND 1 +ve.	15
OD 2 "	12	ND 2 "	16
OD 3 "	7	ND 3 "	15½
OD 4 "	15	ND 4 "	13

(1) <u>Comparison of old and new yeast extracts</u>

Total old yeast extracts OD 1 + OD 2 + ND 1 + ND 2 = 51.

Total new yeast extracts OD 3 + OD 4 + ND 3 + ND 4 = 50½.

There is obviously practically no difference between old and new extract.

TABLE 54.

Effect of Age of digest, yeast extract and plasma, on PDB.

Strain of HILB	Medium	Glucose	Maltose	Arabinose	Xylose	Galactose	Laevulose	Dextrin
T94	OD1	-* -?	+ +	- -	- -	- -	- -	- -
	OD2	- -	- +	- -	- -	- -	- +	- -
	OD 3	- -	+ +	- -	- -	- -	- -	- -
	OD 4	- -	+ +	- -	- -	- -	- +	+ +
	ND1	cont.	+ +	- -	- -	- cont.	± +	+ +
	ND2	- -	- +	- -	- -	- -	- +	+ +
	ND3	+ +	+ +	- -	- -	- -	± +	+ +
	ND4	- -	- +	- -	- -	- -	- +	+ +
T91	OD1	- -	+ +	- -	- -	± +	- -	+ +
	OD 2	- -	+ +	+ +	- -	+ +	- -	- -
	OD3	- -	- -	+ +	- -	- -	- -	- -
	OD4	- -	+ +	+ +	- -	+ +	- -	+ +
	ND1	- -	+ +	+ +	+ +	+ +	- -	+ +
	ND 2	- -	+ +	+ +	+ +	+ +	- -	+ +
	ND 3	- -	+ +	+ +	+ +	+ +	- +	+ +
	ND 4	+ +	+ +	+ +	- -	+ +	- -	+ +

TABLE 54.(contd.).

Strain of HILB	Medium	Glucose	Maltose	Arabinose	Xylose	Galactose	Laevulose	Dextrin
T46	OD1	- -	+ +	- -	- -	- -	- -	+ +
	OD 2	- -	+ +	- -	- -	- -	- -	+ +
	OD 3	- -	+ +	- -	- -	- -	- -	+ +
	OD 4	- -	+ +	- -	- -	- -	- +	+ +
	ND1	- -	+ +	- -	- -	- -	+ +	+ +
	ND 2	+ +	- -	- -	- -	- -	- +	+ +
	ND 3	+ +	+ +	- -	- -	- -	- -	+ +
	ND 4	- -	- -	- -	- -	- -	- -	+ +
T104	OD1	- -	+ +	- -	+ +	- +	- -	- -
	OD2	- -	+ +	+ +	+ +	+ +	- -	+ +
	OD3	- -	+ +	+ +	+ +	- -	- -	- -
	OD4	- -	+ +	+ +	+ +	+ +	- -	+ +
	ND1	- -	+ +	+ +	+ +	- +	- -	- -
	ND2	- -	+ +	+ +	+ +	+ +	- -	+ +
	ND3	- -	- +	± +	- ±	- -	- -	- -
	ND4	- -	+ +	+ +	+ +	- -	- -	+ +

* 5 days incubation † 14 days incubation

cont = contaminated + = acid produced - = no acid produced

± = doubtful reaction.

(2) Comparison of old and new digest.

Total old digest = OD 1 + OD 2 + OD 3 + OD 4 = 43.

Total new digest = ND 1 + ND 2 + ND 3 + ND 4 = $59\frac{1}{2}$.

Observed difference = $(\frac{59.5}{110} \times 100)\% - (\frac{43}{112} \times 100)\% = 15.7\%$

Standard Error of Difference =

$$\sqrt{\frac{p \times q}{n_1} + \frac{p \times q}{n_2}} \%$$

$p = \frac{43 + 59\frac{1}{2}}{222} \times 100\% = 46\%$ therefore $q = 54\%$.

$n_1 = 112 \quad n_2 = 110$

Therefore S.E. of Diff. = $\pm$ 6.7%

The observed difference of 15.7% is more than 2 x S.E.

New digest therefore gives significantly better results than old digest, whether old or new plasma is present.

(3) Comparison of old and new plasma

Using the same method as above, the observed difference in proportion of positive tubes is 7.8%, while the SE. of the difference is $\pm$ 6.7%. This is less than 2 x S.E. so that the difference between the effect of old and new plasma is not statistically significant.

N. Effect of starch and other factors on longevity of cultures

This was investigated by comparing the results

of sub-culture from various media in bijou bottles after the following times of incubation:- 3 days, 7 days, 10 days and 14 days. The media consisted of ordinary broth (Appendix no. 1) with other constituents as specified in Table 55, where the results are recorded.

It will be seen that use of sloppy agar (0.1% New Zealand) made no discernible difference in the longevity of the culture as compared with solid agar (medium 3 compared with 5). The use of 20% human blood gave slight improvement over 6% in solid medium (medium 5 compared with 6) there being 8 out of 8 successful isolations from the former after 3 days, compared with 6 out of 8 in the case of the latter. However, the numbers of 7- and 10-day isolations were the same in the 2 media.

The effect of starch is more obvious, the number of successful sub-cultures from the 3 starch-containing media after 3 days, being only 12 out of 24, 4 of these giving very scanty growth, as compared with 22 out of 24 (only one being very scanty) in the starch-free media. None of the 7-day cultures yielded positive sub-cultures out of a possible 24 as against 10 out of 24 from the starch-free media. This method of adding up total successful sub-cultures is justified, because the two

TABLE 55.

Effect of starch, increased blood content, sloppy agar, and atmospheric conditions.

	Agar conc.	Blood conc.	Starch conc.	Screw-cap.	Anaerobic cult.	+ 10% CO_2.	Strain 269 sub-culture on day no. 3	7	10	14	Strain 272 sub-culture on day no. 3	7	10	14
Medium (1)	0.25%	20% Rabbit	1%	+	-	-	-	-	-	-	-	-	-	.
				-	-	-	+	-	-	-	±	-	-	-
				-	+	-	+	-	-	-	+++	-	-	-
				-	-	+	-	-	-	-	-	-	-	-
Medium (2)	0.25%	20% Human	1%	+	-	-	-	-	-	-	-	-	-	-
				-	-	-	-	-	-	-	±	-	-	-
				-	+	-	±	-	-	-	±	-	-	-
				-	-	+	-	-	-	-	-	-	-	-
Medium (3)	0.25%	20% Human	NIL	+	-	-	+	+	-	-	+	+	+	-
				-	-	-	+	-	-	-	+	±	-	-
				-	+	-	++	-	-	-	+	-	-	-
				-	-	+	+	-	-	-	+	+	+	-
Medium (4)	2.0%	20% Human	1%	+	-	-	++	-	-	-	+	-	-	-
				-	-	-	-	-	-	-	+	-	-	-
				-	+	-	++	-	-	-	+	-	-	-
				-	-	+	-	-	-	-	-	-	-	-
Medium (5)	2.0%	20% Human	NIL	+	-	-	+	+	-	-	+	-	-	-
				-	-	-	+	-	-	-	++	-	-	-
				-	+	-	+	-	-	-	++	-	-	-
				-	-	+	+	+	-	-	+	+	+	-
Medium (6)	2.0%	6% Human	NIL	+	-	-	±	+	-	-	++	+	+	-
				-	-	-	-	-	-	-	+	-	-	-
				-	+	-	+	-	-	-	-	-	-	-
				-	-	+	+	+	-	-	+	-	-	-

Screw-cap = bijou bottle ($\frac{1}{4}$ oz.) with cap screwed on tightly.
Anaerobic = bijou bottle ($\frac{1}{4}$ oz.) with cotton wool stopper, in anaerobic jar.
+ 10% CO_2 = bijou bottle ($\frac{1}{4}$ oz.) with cotton wool stopper, in jar + 10% CO_2.
Where screw-cap, anaerobic culture and CO_2 columns are all negative, it means that aerobic incubation, with cotton wool stopper, was used.

± = very scanty growth; + = scanty growth;

++ = scanty growth; ++ = moderate growth;

+++ = heavy growth. Conc. = concentration.

cult. = culture.

factors, agar concentration and blood concentration have already been shown to be unimportant, and rabbit blood showed little difference from human blood. Apart from these 3 factors, all the media were the same and each had the same number and type of variations of atmospheric conditions.

With anaerobic cultivation, the proportion of successful sub-cultures in starch media after 3 days was 6 out of 6, while in the starch-free media it was 5 out of 6, i.e. there is no significant difference between starch positive and starch negative media. This indicates some degree of protection by anaerobiosis against the effect of starch, up to 3 days of incubation. However, after 7 or more days, no anaerobic cultures yielded positive sub-cultures either from starch positive or starch negative media.

In the case of screw-cap-incubated bottles the figures are:-

3-day cultures with starch:- 2 out of 6 sub-cultures positive.

3-day cultures without starch:- 6 out of 6 sub-cultures positive.

7-day cultures with starch:- 0 out of 6 sub-cultures positive.

7-day cultures without starch:- 5 out of 6 sub-cultures positive.

For CO_2-incubated cultures the corresponding figures are:-

3-day cultures with starch:- 0 out of 6 sub-cultures positive.

3-day cultures without starch:- 6 out of 6 sub-cultures positive.

7-day cultures with starch:- 0 out of 6 sub-cultures positive.

7-day cultures without starch:- 4 out of 6 sub-cultures positive.

The figures for the screw cap and the CO_2 cultures are very similar and both differ markedly from the anaerobic culture figures. It seems probable therefore that the effect of sealing the bottle with a screw cap is simply to trap the CO_2 already in the blood agar medium and give the same effect as incubation with a cotton stopper in an atmosphere of 10% CO_2.

A comparison was also made of ordinary aerobic cultivation (cotton-wool stopper) with anaerobic, the following being the figures for aerobic cultivation:-

3-day cultures with starch:- 4 out of 6 sub-cultures positive (3 very scanty).

3-day cultures without starch:- 5 out of 6 sub-cultures positive.

7-day cultures with starch:- 0 out of 6 sub-cultures positive.

7-day cultures without starch:- 1 out of 6 sub-cultures positive.

A slight difference may be present between the aerobic and anaerobic where starch positive medium is concerned (6 out of 6 3-day sub-cultures being positive with anaerobic culture as against 4 out of 6 in aerobic, but these figures are obviously not significant statistically.

As between aerobic and CO_2 cultures, however, if we add screw-cap and CO_2 figures together, the result is as follows:-

7-day cultures without starch (aerobic):- 1 out of 6 sub-cultures positive.

7-day cultures without starch (CO_2 or screw-cap):- 9 out of 12 sub-cultures positive.

These figures, although small, are in fact statistically significant, the observed difference in proportions of positive sub-cultures being 58%, while the S.E. is ± 25%. CO_2 therefore had an effect in lengthening the life of the culture.

To sum up, the addition of 1% starch to blood agar or sloppy agar markedly decreased the time during which cultures on the medium remained viable. Anaerobic incubation gave some protection against this effect of starch. Where starch was absent, an increased CO_2 content in the atmosphere lengthened the life of the culture, but CO_2 had no such effect when starch was present.

O. Growth in thioglycollate broth: some miscellaneous culture media:

Leopold (1953) claimed that growth of a similar organism occurred in thioglycollate broth, without serum or plasma, if large inocula were used. This was found to occur in the case of HILB, when 5% digest was added to the basic broth. The results of one batch of cultures are shown in Table 56 . Growth was good in media 2 and 3 (v. Appendix numbers 61 and 62) but poor in medium 1. Repeated sub-cultures were successful in the case of 2 and 3, but no growth occurred in sub-culture in medium 1. This was thought to be due to the resazurin contained in it. The addition of 5% plasma to this medium (Oxoid fluid thioglycollate),however, enabled luxuriant growth to occur and to be maintained in sub-culture.

After 3 days, most tubes show an absence of any anaerobic or micro-aerophilic tendency, growth extending to the top of the tube in most cases. Some tubes, however, mainly the Oxoid medium, showed the appearance described in the Table, of growth stopping short of the top of the medium, especially where the more dilute inoculum was used. Other experiments, with plasma thioglycollate tubes and 48 hours incubation, showed that this occurred frequently during the earlier

TABLE 56.

Growth in thioglycollate broth.

Strain	Inoculum dilution	Growth after 3 days at 37°C.		
		Medium 1 Oxoid fluid U.S.P.	Medium 2 0.15% New Zealand agar	Medium 3 0.05% agar
T30	10	++ 1/8T	+++ T	++ T
	10^3	++ 1/3T	++ $\frac{1}{2}$T	+
T91	10	+ $\frac{1}{2}$T	+++ T	+++ T
	10^3	+ $\frac{1}{2}$T	+	+ T
T94	10	$\pm$ $\frac{1}{2}$T M	+++ T	+++ T
	10^3	-	++ T	++ T
T145	10	-	+++ T	+++ T
	10^3	+ $\frac{1}{2}$T	++ $\frac{1}{2}$T	+

Inoculum dilution expressed as the reciprocal of Brown's opacity 1. Approximately 0.2 ml. of each dilution was used as inoculum.

T = growth occurring right to top of medium

$\frac{1}{2}$T = growth occurring up to $\frac{1}{2}$" from top of medium

M = micro-aerophilic zone of maximal growth present.

stages of growth but that, after 4 days, growth invariably extended to the top. The initial stages of growth were therefore being helped by anaerobic conditions. There was never any failure to grow at the bottom of the tube, where fully anaerobic conditions were present.

Anaerobic thioglycollate agar plates also yielded fairly good growth, better than digest agar alone, suggesting that thioglycollate has growth-promoting powers apart from its production of anaerobiosis.

The following miscellaneous culture media were tried during the course of the investigation, in addition to those dealt with in more detail elsewhere in the thesis:-

Human and rabbit blood bijou agar slopes: growth obtained (a)* but not so convenient as blood agar plates.

0.25% blood plasma digest agar, with or without glucose and bromthymol blue: poor growth (a), (Appendix nos. 14 and 15).

Peptone blood broth: (Appendix no.17): growth obtained, but not so useful as blood agar broth (Appendix no. 20).

Maltose blood broth: growth obtained, (Appendix no. 18).

Levinthal's boiled blood agar: not so good as blood agar (a,c); some batches failed to

* *Letters in brackets indicate method used, as given in Section I.*

support growth (Appendix no. 25).

Chocolate agar bijou slopes: same results as boiled blood agar (Appendix no. 27).

Boiled blood broth: results similar to those with blood broth (Appendix no. 28).

Plasma digest glucose phosphate peptone water: no growth (a) (Appendix no. 39).

Steamed plasma digest broth (a), with or without sugar added (d) : growth not so good as in unheated medium and colour change (when indicator present) not so easily read.

Horse serum agar, unsteamed or steamed: no growth (d)(Appendix nos. 41 and 42).

4% human serum agar: poor growth, not maintained in sub-culture (a) (Appendix no. 43).

Loeffler's coagulated serum: no growth with 4 strains (aerobic 37°C.)(d).

C H A P T E R   V.

BIOCHEMICAL REACTIONS.

CHAPTER V. BIOCHEMICAL REACTIONS

Section 1. Sugar fermentation:

Methods:

Two main techniques were used, both involving 2% Evans peptone broth with the addition of 5% human plasma and 5% horse digest (v Appendix, no. 37).

Method (1)

The basic medium consisted of Evans peptone broth (Appendix no. 1). Digest, 5%, and 0.0025% bromthymol blue (dry) were then added, the container well shaken to dissolve the dye and the medium dispensed into 100 ml. bottles and sterilized by steaming for 45 minutes. Sterile sugar solution (Appendix no. 37) was then added to 0.25% concentration, and finally, 5% sterile human "glucose-free" plasma was added (Appendix no. 36) and the medium pipetted with sterile precautions into 5" x 5/8" sterile tubes, in 3 ml. amounts. Preliminary work had shown that no gas was produced in fermentation, so Durham tubes were not used in the tests herein reported. The medium was then incubated aerobically at 37°C. for 48 - 72 hours to test for sterility, and was then ready for use.

At first, human plasma for this medium was taken from the Transfusion Service out-dated blood

(Group O) used routinely in this laboratory for culture media. It was soon discovered, however, that false positive readings were being obtained because of the glucose present in the anti-coagulant used by the Blood Transfusion Service. When special batches of plasma, from citrated blood containing no added glucose, were used, no false positives occurred and this so-called "glucose-free" plasma was then used for all sugar fermentation media. Any naturally occurring blood sugar in the plasma did not affect the tests.

Serum was not used in this medium, since plasma was more readily available in large quantities, in a sterile condition, and experience showed that an equal concentration of serum was not as effective as plasma.

Preparation of inoculum:

Blood agar broth was prepared as in Appendix no. 20. Each strain of HILB was grown for 48 hours at 37°C. on blood agar, and a heavy inoculum transferred with a loop on to the surface of the blood agar and into the supernatant broth. After 48 hours aerobic incubation at 37°C., growth could usually be discerned, mainly on the surface of the blood agar. This growth was then emulsified in the supernatant broth with a sterile Pasteur

pipette and, with the same pipette, 6 - 8 drops of the suspension were added to each tube of sugar medium to be inoculated. Aerobic incubation was then carried out at 37°C. for 12 days, readings being taken at 5 and 12 days.

Reading Fermentation Tests:

A control set of sugar tubes, prepared in exactly the same way as the others, was always included in each batch of tests. The colour change on fermentation, from greenish to yellow, was necessarily rather delicate in many cases, due to poor growth and to the masking effect of the plasma, which brought the original blue colour of the medium to a greenish tinge. Each tube was therefore compared carefully with the corresponding control tube before being read as positive or negative. In method 2, described below, better growth was achieved and colour changes were more easily read.

Method (2)

The same basic medium was used as in method (1), and sugar added in the same way. However, no plasma was added to the medium until the time of inoculation. The inoculum consisted this time of an emulsion in sterile "glucose-free" plasma of a 48-hour 37°C. culture of the strain on blood agar, 0.15 ml. being added to each 3 ml. tube of medium.

This gave a final plasma concentration of 5%, i.e. the same as in method (1).

HILB are very liable to show phases when growth is poorer than usual, possibly due to a poor batch of blood. By the time method (2) was adopted, it had been found by experience that it was no use attempting any sugar reactions unless growth was fully up to standard on blood agar. Under these conditions, a 48-hour culture on a third of a blood agar plate, yielded enough quickly gathered culture, aseptically transferred to the plasma, to give obvious turbidity, after emulsification, of the order of Brown's opacity 2.

For large scale tests, where numerous tubes were to be inoculated, special Pasteur pipettes were drawn which were very thick and long. These were then calibrated by sucking up a measured 0.15 ml. volume of water, previously pipetted into a 3" x ½" tube. A different measured volume of water was used for each pipette, so as to avoid loss by wetting. A mark was made with grease pencil, the length of the "draw" being enough to ensure that this mark was on the narrow portion of the pipette. The pipettes were then sterilised in the hot air oven and used to inoculate the series of sugar tubes with the HILB-plasma emulsion.

When ordinary 1 ml. laboratory pipettes were used, it was found that their comparatively wide external diameter meant that extensive wetting of the outside of the pipette occurred when the tip was placed in fluid, due to contact with the tube wall causing capillary attraction. This led to an undue incidence of contamination when one pipette was used to inoculate numerous tubes of medium. The specially made pipettes were sufficiently narrow to avoid this, and were fast and accurate in operation.

During preliminary work, it was found that the use of 1% sugar often caused inhibition of growth and so 0.25% was used in method (1). However, using method (2) this inhibition was found to be uncommon and so 1% sugar was used in addition to 0.25%, fermentation of each sugar being tested for in duplicate, one tube containing 1%, the other 0.25%. It was occasionally found that growth and fermentation occurred in the 1% tube and not in the 0.25%, and vice versa.

A control set of sugars was always included in each batch of tests, this time "inoculating" plasma without organisms into each tube by the same technique as for the main test. Incubation and reading of the tests was thereafter the same as in method (1).

Use of solid media for fermentation tests:

When 0.25% or 1% sugar was incorporated into blood agar, heavier growth than normal occurred sometimes. When this happened, after 2 - 3 days, a discolouration of the blood took place, the plate eventually turning brown. A drop of bromthymol blue indicator added to the culture with a pipette then changed colour to yellow, indicating the presence of acid. This colour change could be seen even against the colour of the blood agar. Any attempt to incorporate indicator into the medium was useless, as the colour was masked by the blood.

In practice, this method gave much poorer results than the previous methods, as growth was often inhibited by the sugar. Where good growth occurred, however, the results agreed with those obtained by the tube methods.

Sugar blood agar plates with a lining of agar (Appendix, no. 11) were also tried. These gave results slightly better than the unlined, but not as good as the tube method. (It was hoped by this method to protect the acid produced by the colony from the buffering action of the blood proteins, to some extent). Boiled blood agar containing 0.25% maltose gave some positive results, but was not so good as sugar blood agar.

Plasma digest agar (Appendix no. 33) was tried as a basis for sugar reactions and gave growth in some cases, but fermentation was difficult to detect.

Method (3)

A few results were obtained by using 10% serum digest agar with 1% sugar added (v Appendix no. 46), using 0.0025% phenol red as in Amies and Jones' (1957) medium. Thick moist plates were inoculated from 48 hours 37°C. blood agar cultures and incubated aerobically at 37°C. Readings were made at 4 and 7 days, a yellow colour indicating acid production.

Results:

Lactose, saccharose, dulcite, mannite and glycerol were tested by method (1) using the following strains of HILB:-

T numbers 7, 10, 12, 15, 20, 30, 33, 34, 35, 38, 46, 60, 62, 66, 73, 83, 86, 87, 88, 91, 92, 94, 98, 100, 102, 104, 109, 111, 115, 121, 124, 126, 129, 130, 131, 132, 140, 143, 144, 145, 147, 149, 162, 168, 169.

All were negative. It was considered therefore that there was no point in continuing to test the above sugars with further strains and accordingly, lactose, saccharose, dulcite and mannite were dropped from subsequent tests. Glycerol was

continued for some of the tests.

The main results are given in Table 57.

Owing to the variability of results, the same organism giving different reactions with the same sugar at different times, it was necessary to carry out repeated tests and to pool the information obtained. The table thus shows the combined positives of the number of tests shown beside the serial number of the strain, i.e. it represents the maximum number of positives so far obtained by each method. In all probability this apparent variability is due to unknown variations in the composition of the medium, rather than variation of the organism, the number of positive reactions in any batch bearing a strong relationship to the amount of growth occurring, this in turn being related to a particular batch of medium, sometimes the sugar medium and sometimes the batch of blood in the blood agar used to prepare the inoculum.

The number of strains fermenting each sugar is given below:-

	No. of strains fermented	Total number of strains tested
Glycogen	14 (93%)	15
Starch	56 (84%)	67
Arabinose	53 (79%)	67
Xylose	52 (78%)	67
Maltose	51 (76%)	67
Dextrin	50 (75%)	67
Galactose	45 (67%)	67
Laevulose	37 (55%)	67
Glucose	36 (54%)	67
Glycerol	0	45

TABLE 57.

Sugar fermentation results.

T No. of Strain	Times tested	Method	G	Mose	Arab	Xyl	Gal	Laev	Dext	Glyc	St	Glyco
7	2	M1	+	+	+	−	+	+	+	−	+	•
	1	M2 1%	−	+	−	−	−	+	+	•	+	•
		M2 0.25%	−	+	−	−	−	−	+	•	+	•
	1	M3	•	+	•	•	•	•	•	•	•	•
10	2	M1	+	+	−	+	+	+	+	−	+	•
12	2	M1	−	+	+	+	+	+	+	−	+	•
15	2	M1	+	+	+	+	+	+	+	−	+	•
	1	M2 1%	•	•	•	•	•	•	•	•	•	+
		M2 0.25%	•	•	•	•	•	•	•	•	•	+
20	1	M1	−	−	−	−	−	−	−	−	−	•
30	2	M1	+	+	+	+	+	+	+	−	+	•
	1	M2 1%	•	•	•	•	•	•	•	•	•	+
		M2 0.25%	•	•	•	•	•	•	•	•	•	+
33	2	M1	+	+	+	+	+	+	+	−	+	•
	1	M2 1%	•	•	•	•	•	•	•	•	•	+
		M2 0.25%	•	•	•	•	•	•	•	•	•	+
34	2	M1	+	+	+	+	+	+	+	−	+	•
35	1	M1	+	+	+	+	+	+	+	−	+	•
	1	M2 1%	•	•	•	•	•	•	•	•	•	+
		M2 0.25%	•	•	•	•	•	•	•	•	•	+
38	2	M1	−	−	+	+	+	−	−	−	+	•
	1	M2 1%	−	+	+	+	+	+	−	•	+	•
		M2 0.25%	+	+	+	−	+	+	+	•	+	•
46	3	M1	+	+	−	−	−	+	+	−	+	•
60	2	M1	−	−	+	+	−	−	−	−	+	•
62	2	M1	−	+	+	+	−	−	−	−	+	•
66	2	M1	+	+	−	−	−	+	+	−	+	•

TABLE 57 (contd.).

T No. of Strain	Times tested	Method		G	Mose	Arab	Xyl	Gal	Laev	Dext	Glyc	St	Glyco
73	2	M1		+	+	+	+	+	+	+	−	+	•
83	2	M1		−	+	+	+	−	+	+	−	+	•
86	2	M1		+	+	+	+	+	−	+	−	+	•
	1	M2	1%	+	+	+	+	+	+	+	•	+	•
			0.25%	+	+	+	+	+	+	+	•	+	•
87	2	M1		+	+	+	+	+	+	+	−	+	•
	1	M2	1%	−	+	+	+	+	−	+	•	−	+
			0.25%	−	−	+	+	−	−	−	•	−	+
	1	M3		•	•	+	•	•	•	•	•	•	•
88	2	M1		+	+	+	+	+	+	+	−	+	•
	1	M2	1%	•	•	•	•	•	•	•	•	•	+
			0.25%	•	•	•	•	•	•	•	•	•	+
91	3	M1		+	+	+	+	+	+	+	−	+	•
	1	M2	1%	•	•	•	•	•	•	•	•	•	+
			0.25%	•	•	•	•	•	•	•	•	•	+
92	2	M1		+	+	+	+	−	−	+	−	+	•
94	2	M1		+	+	+	+	+	+	+	−	+	•
	1	M2	1%	•	•	•	•	•	•	•	•	•	+
			0.25%	•	•	•	•	•	•	•	•	•	+
98	2	M1		+	+	+	+	+	+	+	−	+	•
	1	M2	1%	•	•	•	•	•	•	•	•	•	−
			0.25%	•	•	•	•	•	•	•	•	•	+
100	2	M1		−	−	−	+	−	−	−	−	+	•
102	3	M1		−	−	+	+	+	−	−	−	+	•
104	5	M1		−	+	+	+	+	−	+	−	+	•
109	2	M1		+	+	+	+	+	−	+	−	+	•
111	2	M1		−	−	+	+	+	−	−	−	+	•
115	4	M1		−	+	+	+	+	−	+	−	+	•
129	2	M1		−	−	±	±	−	−	−	−	+	•
130	1	M1		−	−	−	−	−	−	−	−	−	•
	1	M2	1%	•	•	•	•	•	•	•	•	•	+
			0.25%	•	•	•	•	•	•	•	•	•	+

TABLE 57.(contd.).

T No. of Strain	Times tested	Method	G	Mose	Arab	Xyl	Gal	Laev	Dext	Glyc	St	Glyco
121	1	M1	−	+	+	+	+	−	+	−	+	•
124	1	M1	−	−	+	+	−	−	−	−	−	•
126	2	M1	−	+	+	−	+	−	+	−	+	•
131	2	M1	−	−	−	+	−	−	−	−	−	•
132	3	M1	+	+	+	+	−	−	+	−	+	•
140	3	M1	−	−	+	+	+	−	+	−	+	•
143	2	M1	−	−	−	+	−	−	−	−	−	•
	1	M2 1%	•	•	•	•	•	•	•	•	•	−
		0.25%	•	•	•	•	•	•	•	•	•	−
144	2	M1	−	−	+	+	−	−	−	−	−	•
145	3	M1	−	−	±	+	−	−	−	−	−	•
	1	M2 1%	•	•	•	•	•	•	•	•	•	+
		0.25%	•	•	•	•	•	•	•	•	•	+
147	1	M1	−	±	+	+	−	−	+	−	+	•
149	1	M1	−	+	−	+	−	−	+	−	+	•
162	1	M1	−	−	−	+	−	−	−	−	−	•
167	1	M2 1%	+	+	+	−	+	+	+	•	+	•
		0.25%	+	+	+	−	+	−	+	•	+	•
	1	M3	+	+	+	−	+	+	•	•	+	•
168	1	M1	−	−	+	+	−	−	+	−	+	•
	1	M2 1%	+	+	+	+	+	+	+	•	+	•
		0.25%	+	+	+	+	+	−	+	•	−	•
169	1	M1	•	−	+	+	−	−	−	−	−	•
	1	M2 1%	+	+	+	+	+	+	+	•	+	+
		0.25%	+	+	+	−	−	−	+	•	+	•
	1	M3	•	•	+	+	+	•	•	•	•	•
170	1	M2 1%	+	−	−	−	−	−	+	•	+	•
		0.25%	−	−	−	−	−	−	−	•	+	•
182	1	M2 1%	−	+	+	−	+	+	+	•	+	•
		0.25%	−	+	+	−	±	−	+	•	+	•
	1	M3	•	•	+	•	−	•	•	•	•	•

TABLE 57.(contd.).

T No. of Strain	Times tested	Method	G	Mose	Arab	Xyl	Gal	Laev	Dext	Glyc	St	Glyco
183	1	M2 1%	−	+	+	−	+	+	+	•	+	•
		0.25%	−	+	+	−	+	+	−	•	+	•
	1	M3	•	•	+	•	•	•	•	•	•	•
194	1	M2 1%	−	+	+	−	+	+	+	•	+	•
		0.25%	+	+	+	−	+	+	+	•	+	•
	1	M3	•	•	+	•	+	•	•	•	•	•
205	1	M2 1%	−	+	+	+	−	−	+	•	+	•
		0.25%	+	+	+	+	+	−	+	•	+	•
214	1	M2 1%	+	+	+	+	+	+	+	•	+	•
		0.25%	+	+	+	+	+	+	+	•	−	•
	1	M3	+	•	+	+	•	•	•	•	•	•
216	1	M2 1%	−	+	+	+	+	+	+	•	+	•
		0.25%	−	−	+	+	±	+	+	•	+	•
	1	M3	−	+	−	•	−	−	+	•	•	•
217H	1	M2 1%	+	+	+	−	−	−	+	•	−	•
		0.25%	+	−	+	−	−	−	+	•	−	•
	1	M3	•	•	+	•	•	•	•	•	•	•
217NH	1	M2 1%	+	+	+	−	+	+	+	•	+	•
		0.25%	+	•	−	−	+	•	•	•	•	•
	1	M3	•	•	−	•	•	+	•	•	•	•
220	1	M2 1%	−	+	±	+	+	−	+	•	+	•
		0.25%	−	+	+	+	+	+	+	•	+	•
	1	M3	•	+	+	•	+	•	•	•	•	•
221	1	M2 1%	−	+	+	+	+	+	+	•	+	•
		0.25%	−	+	+	+	+	±	+	•	+	•
224	1	M2 1%	−	−	+	+	+	−	−	•	−	−
		0.25%	−	+	+	−	−	−	−	•	−	+
236	1	M2 1%	+	+	+	+	−	+	+	•	+	•
		0.25%	+	+	−	+	+	−	+	•	+	•
	1	M3	•	+	−	+	•	•	•	•	+	•
237	1	M2 1%	+	+	+	•	+	•	•	•	+	•
		0.25%	−	−	+	+	−	−	−	•	+	•
	1	M3	•	•	+	•	•	•	•	•	•	•

TABLE 57.(contd).

T No. of Strain	Times tested	Method	G	Mose	Arab	Xyl	Gal	Laev	Dext	Glyc	St	Glyco
238	1	M2 1%	-	+	+	+	-	+	-	.	+	.
		0.25%	+	-	-	+	+	+	+	.	+	.
243	1	M2 1%	-	+	+	+	-	+	-	.	-	+
		0.25%	-	-	+	-	+	-	-	.	-	+
251	1	M2 1%	+	+	-	-	+	+	+	.	+	.
		0.25%	+	-	+	-	+	±	+	.	+	.
262	1	M2 1%	+	+	+	-	-	-	+	.	+	.
		0.25%	+	-	+	-	-	-	-	.	-	.
	1	M3	.	.	+	.	.	.	.	.	.	.
266	1	M2 1%	+	+	-	+	+	+	+	.	+	.
		0.25%	-	+	-	+	-	+	+	.	+	.
	1	M3	.	.	-	.	.	.	.	.	.	.
269	1	M2 1%	-	+	+	-	+	+	+	.	+	.
		0.25%	-	+	+	-	+	-	+	.	+	.
275	1	M2 1%	-	+	+	+	+	+	+	.	+	.
		0.25%	+	+	+	+	+	+	+	.	+	.

G = glucose
Mose = maltose
Arab = arabinose
Xyl = xylose
Gal = galactose
Laev = laevulose
Dext = dextrin
Glyc = glycerol
St = starch
Glyco = glycogen

M1 = test done by method 1
M2, 1% = test done by method 2, using 1% sugar
M2, 0.25% = test done by method 2, using 0.25%.
M3 = test done by method 3.
+ = acid produced.
- = no acid produced.
. = no result obtained.

The relatively small number of strains tested against glycogen was due to the fact that an inadequate preliminary experiment using glycogen had given a misleading result, and glycogen was not included in the main series of tests. Later, the striking resemblance of fermenting strains of PPLO, in respect of their fermentation spectrum (v Edward, 1954), to HILB was noted, and it was also seen that one of these fermented sugars was glycogen. A short series of tests was therefore done, using method (2), with the results shown in Table 57 and summarised above. It is clear, in spite of the smaller number of tests done, that glycogen is the most frequently fermented carbohydrate, with starch a close second. Next in order came the 2 pentoses, arabinose and xylose, followed by the disaccharide maltose, the polysaccharide dextrin and last come the 3 monosaccharides galactose, laevulose and glucose. The alcohols, as represented by glycerol, mannite and dulcite, are all negative. The remaining negative sugars tested (lactose and saccharose) are both disaccharides. The relative infrequency with which glucose was fermented is surprising, and contrasts with the ease with which the multiples of the glucose molecule (glycogen, starch, dextrin and maltose) were attacked.

An example of the variability encountered is seen in Table 58, where the results from all strains tested by method (1) 3 times or more are shown. In no case have any two of the tests shown an identical pattern of fermentation.

The variability of the results indicates that any groupings which may be deduced from the results are very tentative, and it is probably not worth while trying to pin down a strain to any particular pattern of sugars, with the proviso that this only applies within the group of fermented sugars, not to the invariably negative lactose, saccharose, dulcite, mannite and glycerol.

A few strains stand out (ignoring those only tested once) for lack of fermentative powers, e.g. T 60, T 62, T 100, T102, T111, T 129, T 124, T 131, T 143, T 144, T 145, T 162, T 170, T 224, which fermented 4 or less out of 8 sugars (not counting glycogen). Those fermenting 2 or less after 2 or more tests are T 100, T 129, T 124, T 131, T 143 (this does not even ferment glycogen), T 144 and T 145.

TABLE 58.

Variability of sugar reactions.

Strain	No. of test	G	Mose	Arab	Xyl	Gal	Laev	Dext	St
T46	1	+	+	-	±	-	+	+	+
	2	-	-	-	+	-	-	-	-
	3	+	+	-	-	-	+	+	.
T91	1	+	+	+	+	+	-	+	+
	2	-	-	+	+	-	-	-	-
	3	+	+	+	+	+	+	+	+
T102	1	-	-	+	+	-	-	-	-
	2	-	-	-	-	-	-	-	-
	3	-	-	+	+	+	-	-	-
T104	1	-	-	+	+	-	-	+	+
	2	-	-	+	+	-	-	-	+
	3	-	-	+	-	+	-	+	+
	4	-	-	-	+	-	-	-	-
	5	-	+	+	+	+	-	+	.
T115	1	-	+	±	+	-	-	+	+
	2	-	+	+	-	+	-	+	+
	3	-	-	-	+	-	-	-	+
	4	-	-	+	+	-	-	-	+
T132	1	+	-	+	+	-	.	+	+
	2	-	-	-	-	-	-	-	-
	3	-	-	+	+	+	-	+	+
T140	1	-	-	-	+	-	-	-	-
	2	-	-	-	+	+	-	+	-
	3	-	-	+	+	+	-	+	+
T145	1	-	-	±	±	-	-	-	-
	2	-	-	-	+	-	-	-	-
	3	-	-	-	.	-	-	-	.

Abbreviations as in Table 57.

Section 2: Biochemistry, other than fermentation.

Methods and Results:

Indole Test:

Plasma digest broth was used for this test, a heavy inoculum from 48-hour 37°C. aerobic blood agar broth cultures being added and incubation for 4 days at 37°C. carried out. The test was done in the usual manner by layering Ehrlich's Indole reagent on to the culture and leaving for 10 minutes. The following 44 strains were tested:- T numbers 7, 10, 12, 15, 30, 33, 34, 35, 38, 46, 60, 62, 66, 73, 83, 86, 87, 88, 91, 92, 94, 98, 100, 104, 109, 111, 129, 130, 102, 115, 121, 124, 126, 131, 132, 140, 143, 144, 145, 147, 149, 162, 168, 169.

All were negative. E. coli, grown in the medium, gave a good positive reaction after 4 days incubation.

Nitrate reduction, Voges-Proskauer and Methyl Red Tests:

Attempts were made to test for the first by growing in nitrate medium (Kauffmann, 1954), superimposed on blood agar, and for the last 2 tests in glucose phosphate peptone water (Mackie and McCartney, 1953) superimposed on blood agar. Uniformly negative results for nitrate reduction were obtained after 5 days incubation, although

sub-culture after 4 days showed that the inoculum was at least viable, if not growing. The same strains as were tested for indole were all examined by this method, but found to be negative. E. coli tested in this medium gave a positive reaction when tested after 5 days incubation.

Methyl red tests in the above-mentioned medium were all negative for the same 44 strains already tested, but Voges-Proskauer tests showed some positive results. Controls, however, gave unsatisfactory results and it was decided to try to produce a massive inoculum of viable HILB cells, free from plasma, etc., which would be heavy enough to produce the required reaction, without actual growth occurring. Six 1% and six 0.25% maltose digest broths were therefore inoculated by method (2) (Chapter V, Section 1) with each of a few selected strains. Heavy growth resulted after 48 hours at 37°C. The contents of the 12 tubes for each strain were then pooled and centrifuged, the deposit being resuspended in a small amount of broth to give a thick suspension. Two drops of this added to 0.5 ml. of glucose phosphate peptone water, for methyl red and V.-P., and to nitrate medium for nitrate reduction, gave a dense suspension of approximately Brown's opacity 5. These tubes were then incubated for 4 days, for V.-P. and

methyl red, and 5 days for nitrate reduction. Readings were made by the methods recommended by Kauffmann (1954). The results are seen in Table 59. The coliform bacilli and the Klebsiella strain were grown in the glucose phosphate peptone water before testing.

Table 59. Methyl Red, Voges-Proskauer and Nitrate Reduction

Strains	Methyl Red	Voges-Proskauer	Nitrate Reduction
T 30	-	-	+
T 46	-	-	+
T 91	+	-	+
T 94	+	-	+++
T 281 (H. influenzae)	-	-	+++
H. canis (NCTC 8540)	-	-	+++
Coliform bacilli controls 1	-	+++	+++
" 2	+	±	+++
" 3	+	-	+++
Klebsiella 264 (1)(K67)	-	++	+++
Blank tube	.	.	-

Methyl Red:-

+ = positive

- = negative

. = not done

V.-P. and nitrate reduction tests:-

+++ = bright pink
++ = moderate pink
+ = faint pink
± = doubtful
- = negative
. = not done

NCTC = National Collection of Type Cultures.

Phosphatase production:

Two methods were used for this test, phenolphthalein diphosphate being added either to Levinthal's boiled blood agar plus 0.25% maltose or to plasma digest broth, in 0.01% concentration, as used by Barber and Kuper (1951). Preliminary results indicated that certain strains, (693, 623, 839, 880, 239 and 837) gave positive results by one or other method (v Table 60). Heavy inocula from 48-hour cultures were used and the tests read after 48 hours incubation in both cases.

In the tube method, 10 drops 2N Na_2CO_3 were added to 3 ml. medium and the colour noted. Pale pink was taken as +, pink as ++, violet pink as +++, and very pale pink as negative. Blood agar sub-cultures were carried out before adding the alkali, and any failing to show growth were excluded. In the plate technique, the cultures were exposed to ammonia vapour and the colour of the colonies noted. In practice, however, it was soon found that the colour differentiation was too poor to give reliable results.

The plate method was applied to 17 strains, T numbers 7, 12, 15, 30, 33, 34, 35, 38, 46, 60, 62, 66, 73, 83, 86, 87, 88, 91, and the tube method to 44 strains (same as in indole test) but no definite positive could be claimed for any of these.

TABLE 60.

Phosphatase production.

Strain	Tube	Plate
693	Pale pink +	++
623	violet pink +++	-
839	no growth	+
880	violet pink +++	++
239	contaminated	++
837	pink ++	+
control	very pale pink	•

+ on plate = faint pink on exposure to NH_3.

++ on plate = definite pink on exposure to NH_3.

- on plate = colony remains grey on exposure to NH_3.

Urease production:

Urea and phenol red were added in the same concentration as in Christensen's medium (Christensen, 1946), to "glucose-free" human plasma digest broth. N/10 HCl was added, enough to make the final reaction yellow with a very faint tinge of pink (pH 6.9). Six drops of 48 hours blood agar broth culture were added and readings made after 2 and 5 days incubation. Proteus gave a strong violet-pink reaction in this medium. The viability of the HILB after 2 days incubation was found to be good in most cases.

The same 44 strains as already listed under Indole Test were tested, and all were found to be negative.

Gelatin Liquefaction:

Nutrient gelatin was superimposed on blood agar in a tube and incubated for 2 days before inoculation with 6 drops 48-hour blood agar broth culture of HILB. The medium was checked for viability of the inoculum after 2 days incubation at 37°C. using a few selected strains. Incubation was carried out for 3 weeks at 37°C. the tubes placed in the refrigerator overnight and read. Thirty strains were tested, T numbers, 7, 10, 12, 15, 20, 30, 33, 34, 35, 38, 46, 60, 62, 66, 73, 83, 86, 87, 88, 91, 92, 94, 98, 100, 104, 109, 111, 115,

129, and 130. T 109 was contaminated, T111 showed slight liquefaction after 3 weeks, the remainder were negative.

Peroxidase and Catalase Production:

Fifteen strains were grown aerobically on 5% human serum digest agar for 48 hours at 37°C. A little of the culture was emulsified in a loopful of water on a slide and benzidine-acetic acid-H_2O_2 mixture added (1 loopful). The medium was also tested by adding a loopful of the mixture to the surface of the agar and watching for a blue stain. The results are given in Table 61.

Table 61. Peroxidase and catalase reactions of 15 strains.

Strains	Peroxidase	Catalase	Strains	Peroxidase	Catalase
T 30	-	-	T 98	+	-
T 91	-	-	T 130	+	-
T 94	-	-	T 143	+	-
T 145	-	-	T 169	-	-
T 15	+	-	T 224	-	-
T 33	-	-	T 243	-	-
T 35NH	-	-	Control staph.	+	+
T 87	-	-			
T 88	-	-	Culture medium control	-	-

T 98, T 130 and T 143 were re-tested, using a dilute inoculum for culture and were again positive. T 15 could not be re-tested, as it failed to grow.

All the strains were catalase negative. The serum agar on which they were grown was both peroxidase and catalase negative.

C H A P T E R VI.

SEROLOGY.

CHAPTER VI SEROLOGY

Section 1 Production of antigens and antisera:

Production of antigens

Agar-lined maltose blood agar plates were prepared as in Appendix (no. 11), care being taken to use really hot agar (80 - 90°C.) for lining the plates, and to measure the amount with a 10 ml. sterile pipette. Three ml. was the best amount to give the maximum protection of the antigen from blood pigments and proteins, with minimum chance of inhibition of growth. To make sure that the agar-lining was constant in thickness over the plate, the simple expedient was adopted of leaving the plates in situ after pouring the blood agar and not moving them again until the lining was added. The effect of this was to counteract any unevenness in the bench or plate, the surfaces of the blood agar and agar lining both remaining horizontal until setting was complete. After pouring, the plates were incubated 2 days at 37°C., partly to test for sterility, partly to dry (open drying was not always practicable, owing to shortage of staff and incubator space) and partly to allow diffusion of growth factor from the blood through the agar lining.

The method of inoculation was important, so as to ensure that the maximum amount of inoculum

was spread over the 5 - 6 plates used for each strain, with the minimum amount of contamination.

Forty-eight-hour blood agar cultures were emulsified in broth, or blood agar broth cultures were made as for sugar fermentation tests (method (1)). Three to four drops of this culture were added to each plate by an assistant, the plates then being quickly spread before there was time for the fluid to soak in, with flamed bent Pasteur pipettes, one being used for 2 - 3 plates at a time. After incubation for 48 hours, the plates were examined and if only a few colonies were seen, these were quickly spread over the remaining surface of the plate with a loop. If growth was confluent, or nearly so, they were left untouched. In each case a further 2 - 3 days incubation, depending on the state of development of the culture, was allowed.

Harvesting was carried out with first a large and thick wire loop to gather the culture into the centre of the plate and then a small loop to collect it and transfer it to a bijou bottle of saline containing some sterile Grade 9 ballotini.

Production of antisera

Saline suspensions of HILB strains were prepared as described above. The strains were chosen so as to be representative of the various

clinical groups. After shaking for 1 hour, the suspensions were standardised to opacity 4 or 5. The first batch of rabbits was immunised with opacity 5 suspensions, and 2 deaths occurred, which may have been related to the large dose of HILB given. For the second series, therefore, opacity 4 antigens were used.

Large, not too old, rabbits were used when possible, but the shortage of these animals sometimes necessitated the use of immature small rabbits. After a preliminary bleeding of 7 - 10 ml. to produce a base-line control serum, 0.1 ml. of suspension was injected intravenously into an ear vein. The antigen was unheated and unpreserved but the majority of organisms must have been non-viable in view of the length of incubation on sugar-containing medium.

After 3 - 4 days, a second injection was given, and the doses were stepped up gradually as follows:- 0.1 ml., 0.2 ml., 0.4 ml., 0.6 ml., 0.8 ml., 1.0 ml. Before the 4th or 5th injection a sample of blood was taken to test for antibody, and if the titre was adequate, 40 - 50 ml. blood was obtained from the ear veins.

In bleeding rabbits, it was found helpful to have them thoroughly warmed up beforehand, and, after shaving the ear and rubbing with a little

benzol, to coat the skin with melted wax to prevent the blood wetting the skin and hairs and becoming contaminated. A longitudinal incision with a new safety razor blade was made in the marginal ear vein, while stretching the ear over a matchbox. The use of this type of incision made healing rapid and complete, so that within a few weeks it was not possible to tell that the vein had been opened. To avoid the production of fatty sera, which were unsuitable for precipition tests becaus of their opacity, bleeding was done several hours after feeding. Courses of immunisation generally lasted about 4 weeks, but in some cases it took longer. However, it was feared that prolonged courses might give rise to excessive cross reactions by the resulting serum, and in any case, supplies of antigen were very limited, so that in practice, the courses did not exceed 6 weeks in length. All rabbits were labelled with a numbered aluminium tag, fastened round a hind leg, so that confusion could not occur between different rabbits.

After separating the serum from the clot, it was centrifuged and stored in the refrigerator after adding 0.08% sodium azide as preservative. Samples of serum were also freeze-dried, in case contamination or loss of the liquid serum occurred.

All sera were controlled before use by testing

a pre-inoculation sample against the homologous antigen. No positive reactions were given by these control sera with precipitation or slide tests but there existed a normal level of complement-fixing activity, which varied with the amount of antigen and complement used. Unheated sera were used for agglutination and precipitation tests, but before performing complement-fixation tests the serum, either undiluted or diluted with saline, was inactivated for half an hour at 56°C.

Section 2:

Agglutination Tests:

Great difficulty was experienced in performing agglutination reactions, owing to the rough suspension normally obtained by any method of culture yet tried. Although colonies do not appear to be rough, having a creamy smooth consistency, and appearing to emulsify quite easily, close inspection with naked eye or hand lens invariably showed the granular quality of the suspension. When such a suspension was allowed to stand, rapid sedimentation occurred, leaving an almost clear supernatant.

Various suspending fluids and methods of suspension were tried, e.g. distilled water, 0.4% saline (Robinson and Peeney, 1936), 1.5% saline (Keogh <u>et al</u>., 1938), broth, N/250 NaOH (Robinson and Peeney, 1936) and heating at 60°C. (Ewing, 1933; Scott, 1923) with no success. Shaking at 500 cycles / minute with Grade 9 ballotini did render suspensions smooth after sufficient time (about 1 - 2 hours) but only at the expense of many disrupted organisms. Even after shaking had produced a smooth suspension, however, if the tube was allowed to stand overnight and the resulting deposit re-suspended, the rough character of the organism re-asserted itself and shaking had to be repeated for about 10 minutes before reasonable

smoothness was regained. In many cases, a suspension so obtained could not be agglutinated by any of the sera, even the homologous one, suggesting that disruption had proceeded to the extent of removing most of the surface antigen. In other cases, the roughness persisted and no results could be obtained.

The technique finally adopted for preparing antigens for agglutination was as follows:-

Cultures were set up, incubated and harvested as already described, except that 0.5% formalin was added to the suspension. The antigens were then shaken with Grade 9 ballotini (occupying roughly half the volume of the suspension) for 1 hour at 600 cycles per minute and stored in the refrigerator. Just before use, the suspensions were re-shaken, 10 minutes for slide, 1 hour for tube agglutination antigens. The slide antigen was then adjusted to a reasonable density (about Brown's opacity 6) and was ready for use.

To perform slide agglutination, 1 loopful of antigen was mixed on a slide with 1 loopful of undiluted serum and the mixture rocked to and fro for a few minutes before reading the result. Controls consisting of antigen and saline were put up at the same time.

For tube agglutination, after the shaking already mentioned, the suspension was diluted with

formol saline to a density of between Brown's opacity 2 and 3 and centrifuged at 1500 r.p.m. for 5 minutes in an angle centrifuge. This deposited most of the larger agglutinating particles and left a smooth turbid supernatant, which was finally adjusted to Brown's opacity 2 and used for the tests.

For the tests itself, serial dilutions of serum (preserved with 0.08% sodium azide) were made in 0.2 ml. volumes of normal saline, in 3" x ½" tubes. 0.2 ml. antigen was added to each tube and the mixture transferred to narrow agglutination tubes and incubated 50°C. for 4 hours, the tests then being read.

Slide agglutination results:

Table 62 shows the results of slide agglutination tests done on 24 strains, 1 of which was H. influenzae (T 281) and the remainder HILB. Four strains were so rough that no readings could be made (T 91, T 92, T 130, T 143). T 66 serum was obtained post-mortem from a rabbit, was too opaque for use in precipitation tests, but gave results in the slide agglutination test.

Several cross reactions were noted between T 30, T 46, T 66, T 94 and T 145, in both vertical and horizontal directions. These 5 were therefore grouped together in Table 63, in which serum T 34 has been omitted, as it fails to react with

TABLE 62.

Slide agglutination results.

Antigen	Serum							
	T30	T34	T38	T46	T62	T66	T94	T145
T30	+	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-
38	-	+	+++	-	±	-	-	-
46	++	-	-	+++	-	+++	+	++
60	-	-	-	-	-	±	+	-
62	-	-	-	-	++	-	+	-
66	-	-	-	+	-	++	-	-
91	rough							
92	rough							
94	-	-	-	-	-	-	++	+++
98	-	-	+	-	-	-	-	-
100	+	-	-	-	-	++	-	+
104	-	-	-	-	-	-	-	++
109	-	-	-	-	-	-	-	-
111	-	-	-	-	-	-	-	-
115	-	-	-	-	-	-	-	-
119	-	-	-	-	-	-	-	-
121	-	-	-	-	-	+	-	-
129	-	-	-	-	-	-	-	-
130	rough							
131	-	-	-	-	-	++	+	-
143	rough							
145	-	-	-	++	-	-	++	++
281 (*H. influenzae*).	-	-	-	-	-	-	-	-

\+ = fine granulation, just discernible.

+++ = coarse rapidly appearing agglutination.

++ = intermediate between + and +++

± = doubtful positive agglutination.

\- = no agglutination.

TABLE 63.

Slide agglutination groupings.

Clinical Group	Antigen	Serum						
		T38	T30	T46	T66	T94	T145	T62
PC	T 38	+++	-	-	-	-	-	±
AN	98	+	-	-	-	-	-	-
PP	30	-	+	-	-	-	-	-
AN	46	-	++	+++	+++	+	++	-
PC	60	-	-	-	±	+	-	-
PP	66	-	-	+	++	-	-	-
PM	94	-	-	-	-	++	+++	-
AN	100	-	+	-	++	-	+	-
PM	121	-	-	-	+	-	-	-
PM	131	-	-	-	++	+	-	-
PP	145	-	-	++	-	++	-	-
GC	62	-	-	-	-	+	-	++
PC	34	-	-	-	-	-	-	-
AN	104	-	-	-	-	-	-	-
PC	109	-	-	-	-	-	-	-
GC	111	-	-	-	-	-	-	-
G	115	-	-	-	-	-	-	-
PM	119	-	-	-	-	-	-	-
G	129	-	-	-	-	-	-	-

its homologous antigen. T 38 and T 98 are grouped together, T 30, T 46, T 66, T 94, T 145, T 60, T 100 are similar and T 62 forms a third group on its own. The clinical groups from which these come are seen in Table 63 also. Three out of 9 in the 2nd. group are PP (puerperal pyrexia), 0 out of 2 in the 1st., 0 out of 1 in the 3rd., and 0 out of 7 untyped strains. Group 2 therefore contains 33% of PP strains as against less than 10% in the other groups, a difference which is too small to be significant.

Slide agglutination tests carried out with 12 HILB sera against T 281, an H. influenzae strain isolated from a case of vulvo-vaginitis in a young girl, were all negative.

Tube agglutination results:

This technique was even more unsatisfactory than the slide agglutination, and only 3 sera showed definite results with their homologous antigens, namely T 94 and T 46, which agglutinated to at least 1/160, and T 62 which agglutinated to 1/40. In all 3 cases the pre-inoculation serum was tested at 1/10 and 1/20 and was negative. No further work was done using this technique.

tubes 1 - 8 of the appropriate row and 0.2 ml. saline was added to tubes 1 - 7 of each row, 0.4 ml. to tube 8 (antigen control) and 0.6 ml. to the cell control. The whole series was then incubated $1\frac{1}{2}$ hours at 37°C. in the incubator.

Haemolytic system was prepared as for the Wassermann reaction. Rabbit anti-sheep-red-cells immune body, in excess, was added to 3% washed sheep red cells and the system sensitised at 37°C. for 1 hour. 0.4 ml. was added to every tube in the test at the end of its $1\frac{1}{2}$ hours incubation, and the tests incubated again for 1 hour at 37°C. The end-point was taken as the highest dilution of complement in which lysis was complete. The titre of complement was expressed in terms of the initial dilution of complement at the end-point. The minimum haemolytic dose (MHD) for each antigen was thus known. In fact, they were all 1/20 except one (T 145) which required 1/30 complement for complete lysis.

Antigen titration:

Serial dilutions of each antigen,(T 30, T 38, T 46, T 62, and T 145) were made in 0.2 ml. volumes, ranging from undiluted antigen to 1/16. 0.2 ml. undiluted antigen was placed in the 6th. tube (antigen control). The 7th. tube in each row was the serum control. 2 MHD complement (0.2 ml. of

1/10 complement, or 1/15 in the case of T 145) were added to each tube (7 tubes in each of 5 rows). Homologous serum diluted 1/16 with normal saline was then added in 0.2 ml. amounts to each tube except the antigen control. Finally, the volume of the 2 control tubes were brought up to 0.6 ml. by adding 0.2 ml. of saline. The test was then treated in the same way as the complement titration. End-points were read where the tube was approximately 50% haemolysed (partial haemolysis). 0.2 ml. of antigen of end-point dilution was referred to as 1 complement-fixing (CF) dose. Two doses were obtained by doubling the concentration, keeping the volume constant.

Serum titration:

Serial dilutions of each serum were set up in 0.2 ml. volumes ranging from 1/8 to 1/64. As each serum was being titrated against 5 antigens there were 5 rows of tubes for each serum. Two CF doses of antigen were added to each tube in the appropriate row, and 2 MHD complement was added to every tube. Controls comprised 5 antigen controls with 2 CF doses in each, and 5 serum controls (in 1/8 dilution). Incubation and addition of haemolytic system was the same as in previous tests. End-points were read in the same way as for the antigen titration.

Preliminary tests had shown that the pre-inoculation sera in all cases had CF titres of less than 1/4 of that of the immune serum.

The results are shown in Table 64 . These are not impressive, the specificity of each serum being indicated by only 1 or 2 tubes in most cases. T 38 showed some degree of specificity, there being at least a 4-fold difference between the homologous and heterologous titres. T 30 showed a consistent 2-fold or greater difference. T 46 showed an 8-fold difference with all except T 145. This compares with the slide agglutination results, where T 145 and T 46 came into the same group. T 62 showed a 4-fold difference from T 46 and T 145, but only a 2-fold from T 30 and T 38. T 145 failed to show any specific titre and this compares with the results in precipitation tests, where T 145 was found to be an unsatisfactory serum.

Attempts were made to perform mass CF tests, but limitation in the supply of antigens made it impracticable to titrate every one and the result was a lack of comparability in the tests which proved so serious that no reliance could be placed on the results and further attempts at serological analysis by this method were abandoned. It must be emphasised that the production of reasonable quantities of even one antigen was a formidable

TABLE 64.

Complement-fixation test for 5 sera and antigens.

Serum	Antigen	Serum dilution (reciprocal)				Ant. C	S-C	Titre (reciprocal)
		8	16	32	64			
T30	T 30	O	O	AC	C	C	C	16
	T 38	O	C	C	C	C		8
	T 46	P	C	C	C	C		8
	T62	P	C	C	C	C		8
	T145	AC	C	C	C	C		<8
T38	T 30	O	AC	C	C		C	8
	T 38	O	O	P	AC			32
	T 46	C	C	C	C			< 8
	T 62	P	C	C	C			8
	T145	C	C	C	C			< 8
T46	T 30	P	C	C	C		C	8
	T 38	P	C	C	C			8
	T 46	O	O	O	P			64
	T 62	P	C	C	C			8
	T145	O	P	AC	C			16
T62	T 30	O	O	P	AC		C	32
	T 38	O	O	P	AC			32
	T 46	O	O	AC	C			16
	T 62	O	O	O	tr			64*
	T145	O	O	AC	C			16
T145	T 30	O	AC	C	C		C	8
	T 38	O	C	C	C			8
	T 46	AC	C	C	C			<8
	T 62	P	C	C	C			8
	T145	P	C	C	C			8

O = haemolysis absent
tr = haemolysis a trace
P = haemolysis partial
AC = haemolysis almost complete
C = haemolysis complete
Ant. C = antigen control
S-C = serum control
* = an extension of the test showed that the titre was no higher.

undertaking, in view of the very small amount of growth obtainable, even under the best conditions, while whole batches were frequently spoilt by contamination due to prolonged incubation of a rich, non-sterilisable medium.

CF tests were carried out against H. influenzae (T 281), H. canis (NCTC 8540) and H. pertussis (NCTC 6988) antigens, prepared in a similar way to HILB antigens except that no shaking was necessary. Levinthal's medium was used for the H. influenzae, and blood agar for the H. canis and H. pertussis. Sera T 30 and T 34 were used against all these. H. canis antigen proved anti-complementary, and no further tests were done. H. influenzae and H. pertussis gave no fixation either with these sera or with T 38, T 46, T 62, T 94 or T 145 sera.

Section 4:

Precipitation Tests:

When it was realised that the technical difficulties involved in agglutination and CF tests were too great for large-scale testing, Lancefield's streptococcal grouping method was tried (Lancefield, 1933) using the HCl extraction technique as described in Mackie and McCartney (1953). Specific precipitation was obtained with the extracts and it was decided to concentrate the rest of the serological study on this method. The problem of obtaining enough antigen was still there, but by the use of narrow bore tubes specially made for the purpose and special finely drawn Pasteur pipettes, demands upon both antigen and serum were kept to a minimum.

Production of antigenic extract:

The antigen was grown and harvested as already described, plain unformolised saline being used. It was found that the amount of culture needed to make 0.5 - 0.8 ml. good antigenic extract was obtained from 3 - 4 whole well-grown plates (4"), this number being increased if growth was poor, or if contaminants reduced the useful area of the culture. Prolonged centrifuging at 3 - 4000 r.p.m. was necessary in many cases in order to produce the absolutely clear solution

which was necessary for reliable results. Extracts were stored in the refrigerator in rubber-stoppered 3" x ½" tubes and were preserved by the addition of 1 drop per ml. of 3.2% sodium azide solution, giving a final concentration of approximately 0.08%. These extracts lasted for many months without obvious alteration in their potency.

Technique of precipitation tests:

Glass tubing of 1.5 mm. bore was washed by sucking through hot detergent solution with the water pump, rinsing in tap-water and drying with alcohol and ether. The outside of the tube was then polished with a damp cloth. The tubes were cut into 4" lengths and one end of each sealed off in a flame. The resulting tube was used for precipitation tests. Pasteur pipettes drawn from ¼" diameter tubes were used to introduce approximately 1 drop of serum into the bottom of one of the special tubes described above. A similar quantity of antigen was then layered carefully over the serum, again using a specially fine Pasteur pipette. The tests were allowed to stand for 1 hour at room temperature and then read. A positive result was denoted by a disk of precipitation at the interface, as shown in Fig. 33. When a positive result was obtained, serial dilutions 1/2, 1/4, 1/8 and 1/16 were made of the serum in

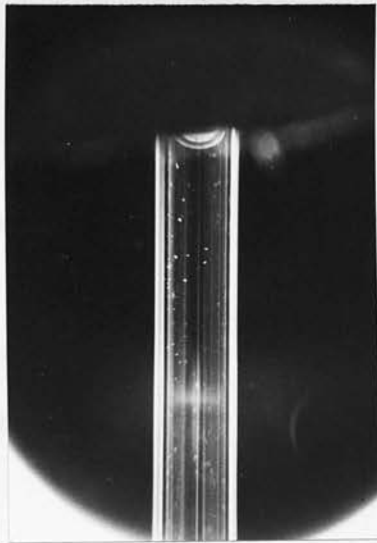


Fig. 33 Precipitation reaction between T238 anti-serum and homologous antigen. X 5.

normal saline, and the test repeated, taking particular care to avoid mixing antibody and antigen during the layering process. It was found quite possible to produce an interface even with 1/16 serum, but the usual titre of the reaction was 1/8 or 1/4 with homologous antigen.

In reading the tests it was necessary to have light coming obliquely from behind the tube, with a dark background to the tube itself. Daylight was normally used, but an electric viewing box designed for reading agglutination reactions was also used, when daylight was too weak.

A few sera were slightly opalescent, in spite of bleeding only fasting rabbits. These were improved by Seitz filtration.

Results of precipitation tests:

Table 65 shows the protocol results, with the sera and antigens arranged in groups, showing cross reactions, and the antigens arranged in numerical order within each group. Fifty HILB strains were tested, 2 non-haemolytic organisms resembling HILB, 1 strain of H. influenzae, and 1 of H. canis. The H. influenzae was isolated from a case of vulvovaginitis in a child of 2 (T 281), the H. canis was obtained from the NCTC (number 8540).

Precipitation groups with proposed antigenic numbering are shown in Table 66. It will be seen

TABLE 65.

Protocol of results of precipitation reactions, arranged in rough groups.

Antigen T. numbers	Antiserum T numbers.												
	216	7	214	38	266	170	46	94	62	34	194	238	30
7	2	2	-	-	-	-	-	1	-	-	-	-	-
38	4	-	-	4	-	-	-	-	-	-	-	-	-
104	8	-	4	-	-	-	-	-	-	-	-	-	-
119	8	-	4	1	-	-	-	-	-	-	-	1	-
129	4	1	-	-	-	1	-	-	-	-	-	-	-
131	4	-	1	4	-	1	-	-	-	-	1	-	-
143	1	-	-	-	-	-	-	-	-	-	-	-	-
167	8	1	2	-	2	-	-	1	-	1	-	-	2
170	1	2	-	-	8	2	-	-	-	-	-	2	-
214	8	-	8	-	-	-	-	-	-	-	-	-	-
216	8	-	1	-	-	-	-	-	-	-	8	-	-
266	1	-	-	-	4	-	-	-	-	-	-	-	-
269	4	-	-	-	-	-	-	-	-	-	-	-	-
35NH	1	-	-	-	-	-	8	1	-	-	-	-	-
46	-	-	-	-	-	-	8	1	-	-	-	-	-
66	-	-	-	-	-	-	8	4	-	-	-	-	-
86	-	-	-	-	-	-	2	-	-	-	-	-	-
87	-	-	-	-	-	-	4	1	-	1	-	-	-
94	-	-	-	-	-	-	8	8	-	-	-	-	-
34	2	-	-	-	-	-	-	-	8	8	-	-	-
62	1	-	-	-	.	-	-	-	8	8	-	-	-
100	-	-	-	-	-	-	-	-	8	8	-	-	-
111	-	-	-	-	-	-	-	-	8	-	2	-	-
115	-	-	-	-	-	-	-	-	8	-	2	-	-
194	8	1	1	-	2	-	-	-	-	-	8	-	-
220	-	1	-	1	-	-	-	-	-	-	4	-	-
237	-	-	1	-	-	-	-	-	-	-	-	2	-
238	-	2	-	-	-	-	-	-	-	-	-	16	-

TABLE 65.(contd.).

Antigen T. numbers	Antiserum T numbers.												
	216	7	214	38	266	170	46	94	62	34	194	238	30
30	-	-	-	-	-	-	-	-	-	-	-	-	4
60	8	-	4	-	-	-	8	1	-	-	-	-	-
121	4	2	2	-	-	-	8	1	-	-	-	2	-
145	2	1	-	-	2	1	4	2	-	-	-	-	-
169	4	4	2	1	1	4	4	1	-	1	-	-	-
251	4	1	-	1	-	-	4	1	1	1	-	-	1
182	2	1	-	-	-	-	-	-	-	-	-	2	-
92	8	-	2	-	-	-	-	-	-	-	-	1	8
275	4	1	-	-	2	-	-	-	-	-	-	1	4
183	•	-	-	1	•	-	1	2	1	1	2	-	1
224	2	1	-	1	-	-	-	1	-	1	-	-	1
243	4	2	4	-	2	2	2	-	-	1	4	2	-
91	-	-	-	-	-	-	-	-	-	-	-	-	-
98	-	-	-	2	-	-	-	-	-	-	-	-	-
109	-	-	-	-	-	-	-	-	-	-	-	-	-
130	-	-	-	-	-	-	-	-	-	-	-	-	-
168	-	-	-	-	-	-	-	-	-	-	-	-	-
205	-	-	-	-	-	-	-	-	-	-	-	-	-
217H	-	-	-	-	-	-	-	-	-	-	-	-	-
217NH	-	-	-	-	-	-	-	-	-	-	-	-	-
219	-	-	-	-	-	-	-	-	-	1	-	-	-
221	-	-	-	-	-	-	-	-	-	1	-	-	-
236	-	-	-	-	-	-	-	-	-	-	-	-	-
262	-	-	-	-	-	-	-	-	-	-	-	-	-
281	-	-	-	-	-	-	-	-	-	-	-	-	-
H.influenzae H.canis (NCTC 8540)	-	-	-	-	-	-	-	-	-	-	-	-	-

T35NH, originally faintly haemolytic, became non-haemolytic later.
T217H = haemolytic strain; T217NH = non-haemolytic organism in same specimen

TABLE 66.

Precipitation groups, with clinical origin of each strain.

Antigenic Group	Clinical Group	Serum	1 T216	2 T46	3 T62	4 T194	5 T238	6 T30
		Antigen T No.						
1	PP	7	2	-	-	-	-	-
	PC	38	4	-	-	-	-	-
	AN	104	8	-	-	-	-	-
	PM	119	8	-	-	-	1	-
	G	129	4	-	-	-	-	-
	PM	131	4	-	-	1	-	-
	G	143	1	-	-	-	-	-
	PP	167	8	-	-	-	-	2
	PC	170	1	-	-	-	2	-
	PP	214	8	-	-	-	-	-
	PP	266	1	-	-	-	-	-
	PP	269	4	-	-	-	-	-
2	GC	35NH	1	8	-	-	-	-
	AN	46	-	8	-	-	-	-
	PP	66	-	8	-	-	-	-
	PC	86	-	2	-	-	-	-
	PP	87	-	4	-	-	-	-
	PM	94	-	8	-	-	-	-
3	PC	34	2	-	8	-	-	-
	GC	62	1	-	8	-	-	-
	AN	100	-	-	8	-	-	-
	GC	111	-	-	8	2	-	-
	G	115	-	-	8	2	-	-
4	PP	194	8	-	-	8	-	-
	PP	220	-	-	-	4	-	-
5	PP	237	-	-	-	-	2	-
	PC	238	-	-	-	-	16	-
6	PP	30	-	-	-	-	-	4
1,2	PC	60	8	8	-	-	-	-
	PM	121	4	8	-	-	2	-
	PP	145	2	4	-	-	-	-
	PP	169	4	4	1	-	-	1
	PP	251	4	4	-	-	-	-

TABLE 66. (contd.).

Antigenic Group	Clinical Group	Serum	1 T216	2 T46	3 T62	4 T194	5 T238	6 T30
		Antigen T No.						
1,5	PP	182	2	-	-	-	2	-
1,6	GC	92	8	-	-	-	1	8
	PP	275	4	-	-	-	1	4
Non-specific	PP	183	.	1	1	2	-	1
	PP	224	2	-	-	-	-	1
	PP	243	4	2	-	4	2	-
Reactions negative or insufficient for grouping.	G	91	-	-	-	-	-	-
	AN	98	-	-	-	-	-	-
	PC	109†	-	-	-	-	-	-
	G	130	-	-	-	-	-	-
	PP	168	-	-	-	-	-	-
	PC	205†	-	-	-	-	-	-
	PP	217H	-	-	-	-	-	-
	PP	217NH	-	-	-	-	-	-
	PC	219	-	-	-	-	-	-
	PC	221	-	-	-	-	-	-
	PP	236	-	-	-	-	-	-
	PP	262	-	-	-	-	-	-
	Child	281	-	-	-	-	-	-
		H.influenzae	-	-	-	-	-	-
		H.canis	-	-	-	-	-	-

† = attempts to make antiserum unsuccessful.

. = no test, as antigen finished.

Clinical group abbreviations as in Table 1.

that 28 of the 50 HILB strains tested, together with the non-haemolytic strain T 35 NH, could be placed in one or other of 6 main groups, namely 1, 2, 3, 4, 5 or 6. Of these, 13 were assigned to group 1; 6 to group 2 (including the non-haemolytic strain); 5 to group 3; 2 to group 4; 2 to group 5 and 1 to group 6. Five strains constituted group 1,2, giving comparable reactions with both 1 and 2 sera. One strain alone formed group 1,5 and 2 strains 1,6. Three gave numerous cross-reactions and could not be assigned to any particular group, while 10 (plus the second non-haemolytic strain T 217 NH, 1 H.influenzae and 1 H.canis strain) gave reactions either completely negative or insufficient for grouping. T 98 antigen reacted with T 38, but not with any of the rest of the group sera, even the "master" serum T 216, and so was not assigned to group 1. It was found impossible to produce an immune serum against antigens T 109 and T 205 with the antigen available.

The distribution of clinical groups among the precipitation groups is seen in Table 67. The only example of uneven distribution is seen in the case of the PP group, which is not represented in precipitation group 3, 0 out of 5 group 3 strains being PP while 25 out of 47 of all strains are PP. The numbers are too small for statistical significance, however.

TABLE 67.

Distribution of precipitation groups among clinical groups.

Clinical Group	Precipitation group											
	1	2	3	4	5	6	1,2	1,5	1,6	non-spec.	Total grouped	not grouped
PP	6	2	0	2	1	1	3	1	1	3	20	5
PC	2	1	1		1		1				6	4
AN	1	1	1								3	1
G	2	0	1								3	2
GC	0	1	2						1		4	0
PM	2	1					1				4	0

non-spec. = non-specific.   For clinical group abbreviations, see Table 1.

TABLE 68.

Comparison of agglutination and precipitation groups.

Slide agglutination group.	Strain	Precipitation group	Slide agglutination group	Strain	Precipitation group
1	T30	6	No reaction	T34	3
	T46	2		T104	1
	T60	1,2		T109	Ungrouped
	T66	2		T111	3
	T94	2		T115	3
	T121	1,2		T119	1
	T131	1		T129	1
	T145	1,2			
2	T38	1			
3	T62	3			

Comparison of results of agglutination and precipitation reactions:

The corresponding precipitation group of each strain in the 4 slide agglutination groups is shown in Table 68. There are too few successful slide agglutinations to compare in any detail groups obtained by the 2 different techniques, but a few points emerge on inspection of these groups. T 38 and T 62 appear as separate types by slide agglutination, and precipitation also shows them to be different from each other and from most of the strains of slide agglutination group 1. More striking, however, is that 5 out of the 6 agglutination group 1 strains also have a common antigenic factor (2) by precipitation. It would appear that there is a rough degree of correspondence between slide agglutination, where feasible, and precipitation.

Precipitation tests with patient's serum:

The following patients had a specimen of blood taken for serological testing.

T 262: Case of puerperal pyrexia, temperature over 99°F. on 4 occasions.

HILB isolated in heavy growth.

T 266: Case of PP, temperature 100.8, 99.5 and 99.5°F. on 3 respective occasions.

HILB isolated in heavy growth.

T 273: Case of PP later diagnosed as having a breast

abscess.

HILB isolated in heavy growth.

T 277: Case of PP, temperature 99.4, 99.8, 100.0, 99.4, 99.4 on 5 respective occasions.

True H. influenzae isolated, no HILB.

T 265: Case of PP, temperature 99.0, 99.0, 99.0 on 3 respective occasions.

HILB isolated in heavy growth.

T 279: Case of PP later diagnosed as phlebitis.

HILB isolated in heavy growth.

These sera were all taken within a few days of each other, just before the patient was discharged. Table 69 shows the antigens against which each serum was tested.

Table 69 . Precipitation tests with patients' sera:

Antigen	Group	Serum					
		T 262	T 266	T 273	T 277	T 265	T 279
T 262	1	-		-	-	-	-
T 266	1		-	-	-	-	-
T 269	1			-	-	-	-
T 275	1,6			-	-	-	-
T 66	2	-	-	±	-	±	±
T 100	3	-	±	-	-	-	-
T 220	4	-	-	-	-	±	-
T 238	5	-	-	-	-	-	-
T 30	6	-	-	-	-	-	-

± = doubtful

- = negative

Doubtful reactions were seen with 4 of the sera, but no definite positives could be recorded. Possibly the sera were taken too soon after the infections, or more probably, the brief infection was unable to produce the relatively high antibody level required for a precipitation reaction to be detected.

Anti-haemolysin tests:

An attempt was made to demonstrate anti-haemolysin in the case of 4 sera, T 38 (precipitin titre 1/4), T 62 (1/8), 880 (1/4) and 693 (1/8). The last two were sera from rabbits which had been immunised several months earlier, whose antibody had been boosted by a second course of injections. They were not considered suitable for the main series of tests, as it was feared that non-specific reactions might result from their mode of preparation.

Fresh, unpreserved, unheated serum was used for the tests and 1 drop of serum (undiluted, diluted 1/5, or 1/25 in normal saline) was added to each half of a blood agar plate. When the surface had dried, a 48-hour culture of the homologous strain was inoculated over the area of the drop and on the surrounding medium.

All strains grew normally and produced the usual amount of haemolysis, as judged by naked eye

appearance after 2 - 3 days incubation at 37°C.

The tests were repeated, using 3 drops of serum concentrated on a quarter of the area of the blood agar, and this time inoculating the plate before adding the serum. All 4 strains grew just as well in the serum zone as they did on the rest of the plate, and haemolysis was equally marked on both areas concerned.

CHAPTER VII.

OTHER PROPERTIES, INCLUDING RESULTS OF ANIMAL INOCULATION.

CHAPTER VII. OTHER PROPERTIES OF HILB, INCLUDING RESULTS OF ANIMAL INOCULATION.

Section 1: Animal inoculation

Strains of HILB used were:-

T 30: isolated from a PP case 7/3/57

T 183: " " " " " 23/5/57

T 198: " " " post-menopausal case with vaginitis, 25/5/57

T 238: " " a PC case, 10/6/57.

Inoculation of Guinea-pigs

i. Intra-vaginal:

25/6/57. Twenty-four hour blood agar cultures of T 198 and T 238 were emulsified in plasma maltose broth (Appendix, no. 37) to Brown's opacity 2 (approximately). One to two drops of each were inoculated by Pasteur pipette intra-vaginally into a guinea-pig which had borne a litter 3 weeks earlier. Films and culture were carried out, but were contaminated with Proteus. Daily inspection for 1 week failed to reveal any discharge.

ii. Intra-peritoneal:

27/11/58. Heavy suspensions of 48-hour blood agar cultures of T 30, T 238 and T 183 were made in plasma broth and 1 ml. inoculated intra-peritoneally into guinea-pigs, one for each strain. Rectal temperatures were recorded before and after the

inoculation. These are given in Table 70 . No evidence could be found of any significant rise in temperature following the injection.

iii. Intra-dermal:

0.1 ml. of each of the same suspensions as in ii. was inoculated into the shaved skin of a guinea-pig, all 3 on one animal. No rise in temperature occurred (v. Table 70) and no lesion developed during the ensuing week, daily inspection being made.

Inoculation of rabbits:

i. Intra-vaginal:

12/5/58. A few drops of a 48-hour blood agar broth culture of T 30 were inoculated intra-vaginally by Pasteur pipette into a rabbit that had just borne a litter that same day. Films and culture were made on the 2nd. and 3rd. day after inoculation. Both films showed mixed flora only. The 2nd. day culture gave only Staph. albus and coliform bacilli, but the 3rd. day culture yielded an organism resembling HILB, in moderate numbers. Pure cultures were obtained by picking single colonies.

On blood agar this organism gave less marked haemolysis than T 30. A film of the culture showed a partially Gram positive pleomorphic bacillus. Further culture revealed a tendency to

TABLE 70.

Rectal temperature in °F. of guinea-pigs inoculated intra-peritoneally and intra-dermally with HILB.

Strain		Intra-peritoneal			Intra-dermal
Date	Time	T30	T238	T183	T30,T183 T238
27/11	11 a.m.	102.8	102.0	102.2	99.0
	12.45 p.m.	102.2	100.8	103.0	100
27/11	4.0p.m.	101.8	101.0	103.2	100.2
28/11	7.15 a.m.	102.0	100.6	102.8	99.6
	10.30 a.m.	102.0	101.0	102.2	102.8
	4.0 p.m.	101.8	101.2	102.8	101.6
29/11	7.15 a.m.	101.6	101.0	102.4	100.8
30/11	10.30 a.m.	101.8	101.2	101.8	101.0
31/11	10.30 a.m.	101.6	101.0	102.2	100.6

satellitism round contaminating staphylococcal colonies, although some growth occurred away from these. Microscopic examination now showed very small Gram negative cocco-bacilli, more coccal and smaller than T 30. The organism was then tested for requirement of V factor, as follows:-

A faintly turbid emulsion in broth of a 48-hour blood agar culture of the rabbit organism, T 30 and T 281 respectively was made and the following media inoculated thinly:-

1. 2% peptone agar (ordinary agar, or OA)
2. OA + 1 drop 1/1000 haematin added to area of inoculation (OA + X)
3. OA + 1 drop 1/1000 co-enzyme I added to area of inoculation (OA + V)
4. OA + 1 drop 1/1000 co I and haematin (OA+X+V)
5. Blood agar control.

The results are seen in Table 71.

Table 71. Effect of X and V on rabbit HILB

	Growth after 48 hours at 37°C.				
	OA	OA + X	OA + V	OA+X+V	Blood agar
Rabbit HILB	-	±	-	-	+++
T 281 (H. influenzae)	-	-	-	+++	+
T 30	-	±	-	-	+++

± = very faint growth   + = scanty growth

+++ = good growth   - = no growth

No growth occurred on OA+X+V, unlike H. influenzae, which showed good growth. It seemed therefore unlikely that the satellitism observed had been due to V factor supplied by the contaminant. The experiment had, however, revealed no further difference from T 30, the two organisms behaving in a similar manner under the conditions of the experiment.

In view of the experience with penicillinase on blood agar described in Chapter IV, cultures were deliberately contaminated with the standard staphylococcus, NCTC 6571. Satellitism occurred with the rabbit HILB(but not with T 30) showing that this phenomenon was not due to penicillinase. This was confirmed in a final experiment indicating the difference between the rabbit HILB and T 30.

Crude catalase, dissolved 1 in 10 in phosphate buffer and membrane-filtered, was added to blood agar. Co-enzyme I was added as in the previous experiment. Inoculation with T 30 and the rabbit strain was carried out and additional pin-point inocula of the standard staphylococcus, a penicillinase-producing staphylococcus, E.coli and N. catarrhalis were made so as to impinge on the HILB and rabbit strain inocula. The result is seen in Table 72.

Table 72. Satellitism of rabbit HILB

Strain	Satellitism after 48 hours on blood agar plus					
	Catalase	CoI	NCTC 6571	PR Staph.	*E. coli*	*N. catarrhalis*
T 30	-	-	-	-	-	-
Rabbit HILB	-	-	+	+	+	+

PR = penicillin resistant, i.e. a producer of penicillinase in this case.

It was concluded that the rabbit HILB was not T 30.

A further film and culture from the rabbit 14 days after inoculation, showed a few Gram negative bacilli microscopically, but only *Staph. albus* in culture.

17/12/58. A further intra-vaginal rabbit inoculation was carried out, using strain T 183, the inoculum this time being a heavy emulsion of a 48 hours blood agar culture in 10% rabbit plasma digest broth. The inoculation was performed 2 days after parturition. Films of vaginal washings failed to reveal any organisms resembling HILB either before or after (at 2 days and 4 days) the inoculation.

ii. Intra-venous: (i.v.)

iii. Intra-peritoneal:(i.p.)

iv. Intra-dermal: (i.d.)

Seven rabbits were inoculated with a dense suspension of 48-hour blood agar cultures as follows:

Strain T 30, T 183 and T 238 i.v. into 1 rabbit each (0.4 ml.)

Strain T 30, T 183 and T 238 i.p. into 1 rabbit each (0.4 ml.)

Strain T 30, T 183 and T 238 i.d., all 3 into 1 rabbit (0.15 ml.)

The intra-dermal injections were done on the shaved hindquarters. No lesion developed during the following week. Rectal temperatures were taken frequently for each rabbit and these are shown in Table 73.

An obvious rise of 2°F. occurred in the temperature of the rabbit inoculated with T 183 i.v. after an initial period of at least 2½ hours without any rise. The temperature remained higher than normal for 2 days. T 30 also showed an apparent rise, the temperature averaging 103.5°F. for 5 days after inoculation, as compared with the 2 pre-inoculation readings of 102.4 and 101.1°F., and the average of 102.2°F. for all the control rabbit readings.

The animal inoculated with T 238 i.v. showed an initial rise to 103.8°F., falling next day to

TABLE 73.

Rectal temperatures (°F) of rabbits inoculated with HILB.

Strain		T30 T183 T238	T30		T183		T238	
Date	Time	i-d	i-v	i-p	i-v	i-p	i-v	i-p
15/10	11.0 a.m. 12.45 p.m.	101.4	102.4 101.1	100.8 101.2	102.2 102.0	101.8 102.5	101.6 101.3	101.0 101.0
	3.30 p.m. 5.0 p.m.	[illegible] 101.2	103.2 103.6	101.0 102.6	102.1 104.0	102.3 103.2	101.0 103.8	100.0 101.6
16/10	7.30 a.m. 4.0 p.m.	102.6 102.6	102.8 103.8	102.6 102.2	103.0 103.2	101.8 102.6	102.8 101.8	102.8 103.2
17/10	7.30 a.m.	102.4	103.0	102.4	102.8	102.2	102.2	102.4
18/10	7.30 a.m. 6.0 p.m.		103.6 103.8	101.8 102.4	101.8 102.6	103.0 103.0	102.6 103.0	102.2 102.4
19/10	8.0 a.m. 6.0 p.m.		103.6 104.0	101.8 102.6	102.0 102.4	102.6 103.2	102.8 103.0	102.4 102.8
20/10	7.30 a.m.		103.6	102.8	101.8	102.4	102.8	103.0

Control rabbits

Date	Time	1	2	3	4
16/10	4.0 p.m.	102.2	103.0	101.8	102.6
17/10	7.30 a.m.	102.8	102.2	102.2	101.8
18/10	7.30 a.m. 6.0 p.m.	101.6 102.2	102.2 102.0	101.8 102.4	101.4 102.4
19/10	8.0 a.m. 6.0 p.m.	101.8 102.4	102.6 102.6	102.0 103.0	102.2 102.8
20/10	7.30 a.m.	102.2	102.0	102.4	101.8

i-d = intra-dermal.
i-v = intra-venous.
i-p = intra-peritoneal.

The double line indicates the time of inoculation, just after taking the temperature at 12.45 p.m.

100.8°F. and then rising to 103°F. on the 3rd. day and maintaining a relatively high level till the end of the readings, as compared with the 2 pre-inoculation readings. T 238 i.p. also produced a rise from 101°F. before the inoculation to 103.2°F. the following day, staying well above 102°F. till the end of the readings.

There seems little doubt that mild pyrexia followed the inoculation of T 30 i.v., T 183 i.v. and T 238 i.p., with the possibility of a significant rise with T 238 i.v. as well.

Inoculation of Mice:

Culture from 1 whole blood agar plate after 48 hours at 37°C. was emulsified in broth. Half the emulsion was inoculated intra-peritoneally into a mouse, the other half, with the addition of 3.5% mucin, into another mouse, also intra-peritoneally. This procedure was carried out with the 3 strains T 30, T 183, and T 238, thus accounting for 6 mice. A 7th. mouse was inoculated with 0.3 ml. 3.5% mucin in broth i.p. as a control.

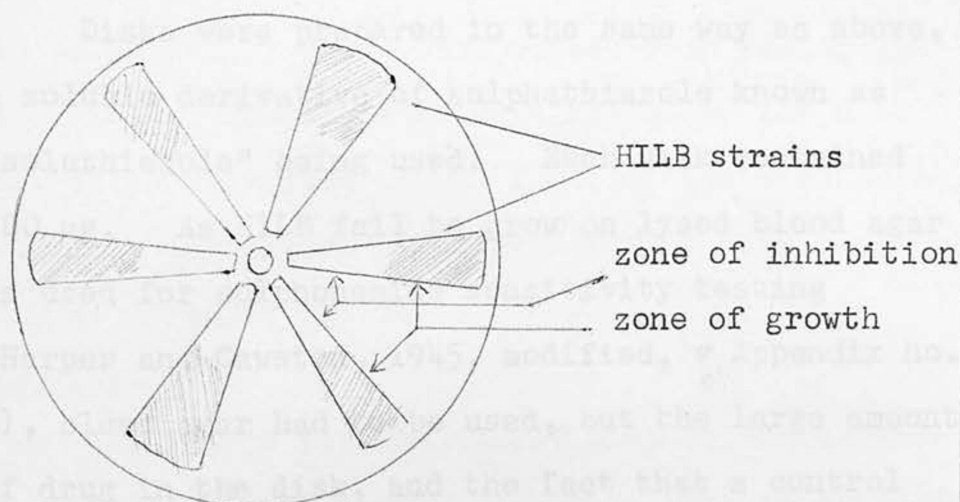
There was no obvious effect following these injections, all the mice remaining lively and continuing to eat normally. After 1 week the 6 test mice were killed and the peritoneal fluid and heart blood cultured. No lesions were seen at the post-mortem examination, and all the organs

appeared normal. The cultures of peritoneal fluid and heart blood were all sterile.

Section 2 Sensitivity of HILB to antibiotics, sulphonamide, and topical agents:

i. Systemic antibiotics:

Filter paper disks 6.5 mm. in diameter (Whatman no. 1) were prepared according to the technique of Gould and Bowie (1952). Zones of inhibition were so large with the majority of antibiotics that it was necessary to use strokes of inoculum radiating from a single central disk on blood agar, instead of the usual method of placing several disks on a plate inoculated with a single organism (v diagram)



The amount of antibiotic in each disk was as follows:-

Chloramphenicol, 25 µg; "sensitive" = sensitive to <10 µg/ml.

Penicillin, 10 units; " = sensitive to <1.0 unit/ml.

Streptomycin, 10 µg; " = sensitive to <1.0 µg/ml.

Tetracycline, 10 µg; " = sensitive to <2.5 µg/ml.

Erythromycin, 10 µg; " = sensitive to <2.5 µg/ml.

Polymyxin, 100 units; " = sensitive to <50 units/ml.

Novobiocin was also tested, by using the 50 µg tablet supplied by the manufacturer, otherwise the technique was as above.

The following strains were tested for sensitivity:- T numbers 34, 35NH, 38, 46, 60, 62, 91, 92, 94, 98, 100, 104, 109, 111, 115, 119, 121, 129, 130, 131, 143, 145.

ii. Sulphonamide:

Disks were prepared in the same way as above, a soluble derivative of sulphathiazole known as "soluthiazole" being used. Each disk contained 100 µg. As HILB fail to grow on lysed blood agar as used for sulphonamide sensitivity testing (Harper and Cawston, 1945, modified, v Appendix no. 3), blood agar had to be used, but the large amount of drug in the disk, and the fact that a control organism gave a wide zone of inhibition, showed that the method was in fact suitable. At first,

all the strains were tested using a heavy inoculum, as normally used for sub-culturing HILB. As all were resistant, the tests were repeated with 6 strains specially plated so as to obtain single colonies in the neighbourhood of the disk. Five of the 6 were still resistant, only one being sensitive.

iii. Topical agents:

Nystatin, acetarsol, "Pruvagol" and gentian violet were tested. Concentrations were decided on the basis of those likely to be attained in the vagina. For acetarsol, 1 SVC tablet (containing 260 mg. acetarsol) was dissolved in 10 ml. water. One suppository of "Pruvagol" was dissolved in 10 ml. water, and gentian violet was used in 1% aqueous solution. In the case of "Pruvagol" (v. Appendix no.70) the gelatin of the capsule had to be melted by using warm water, when making the solution. These agents were tested by the disk method as described above, 1 ml. of solution being added to 100 disks. For nystatin, a disk containing 2,500 units was used.

Results:

All the 22 strains tested with chloramphenicol, penicillin, streptomycin, tetracycline and erythromycin were highly sensitive to these antibiotics. Twenty-one strains were sensitive to novobiocin,

but resistant to polymyxin.

The same strains, with the exception of T 115, were tested with the other agents already described. All the strains were resistant to sulphonamide (heavy inoculum), 5 out of 6 were also resistant when dilute inocula were used. All 21 strains were sensitive to gentian violet, but resistant to nystatin, acetarsol and "Pruvagol".

Section 3: Haemagglutination by HILB:

Method:

One per cent maltose digest broths, without indicator, were inoculated as described in Chapter V, Section 1, method 2, with a plasma emulsion of HILB. In one case, T 111, arabinose was used in the broth instead of maltose, as this strain had not fermented maltose in the past. After 48 hours aerobic incubation at 37°C., the tubes were centrifuged lightly, the deposits thoroughly mixed with the remains of the supernatant, and then used for haemagglutination tests.

A glazed porcelain tile with 12 depressions was cooled on ice until condensation began to appear on its upper surface. To 1 drop of 3% washed red cells (human group O or other species)

previously placed in the depression, 1 drop of HILB deposit was added. The tile was then lifted from the ice and shaken gently until mixing of HILB and red cells was complete. It was then rocked slowly for 2 - 3 minutes. If no agglutination had been seen by then, it was re-cooled and again examined in a similar manner. After reading the results in the cold, the tile was warmed up by holding it some way above a Bunsen flame, until it was at room temperature (approximately), when readings were again taken. Any tile which had shown a positive result in the cold, was then re-cooled and readings again taken. (v. Figs. 34-37).

Method of measuring effect of pH, formaldehyde, heating and mannose:

i. pH:

The HILB suspension was produced as described above, except that several tubes of culture were pooled and centrifuged, the supernatant being discarded. Ten ml. volumes of digest broth were adjusted by adding N/2 NaOH or N/2 HCl, to the various pH levels required. These pH levels were checked with a BDH capillator in all except the pH 9 and 10 tubes, which were measured by universal indicator papers. To 3-4 ml. of broth of each pH level, 4 drops of HILB deposit were added, mixed and centrifuged at about 1000 r.p.m. in an angle centrifuge until the organisms were largely deposited.

The deposit was then assumed to be at the same pH as the broth to which the organisms had been added. These deposits were then tested against 3% washed red cells as before.

ii. Formaldehyde:

To test the effect of formaldehyde, the concentration mentioned by Duguid et al. (1955) was first used. One drop of 10% formalin (4% formaldehyde) was added to 3 drops of suspension prepared as before, this giving a concentration of formaldehyde of 1%. The deposit was then tested by the usual method. The pH of the deposit was 6.0.

Formaldehyde in 2% concentration was next used, as recommended by Duguid (1959). The deposits of 4 strains, T 91, T 94, T 145 and T 198, prepared as above, were made up to 5-6 ml. with peptone broth and 1/3 of its volume of 20% formalin was added. This gave a final concentration of 2% formaldehyde in the suspension, which was then placed in an incubator at 37°C. for 3 hours, centrifuged and the deposit used as already described. As controls, similar suspensions without formalin were kept in the incubator and then tested in parallel with the formolised suspensions.

iii. Heat:

The effect of boiling was tested by using

T 145 suspension prepared in the usual way, the deposit then being brought to the boil over a low Bunsen flame. As soon as boiling occurred, the tube was cooled and the deposit tested as before.

To determine the effect of a lesser degree of heat, the deposits of 3 strains, T 91, T 94 and T 145 were washed once with 5-6 ml. peptone broth to remove plasma and then suspended in about 0.5 ml. peptone broth in a 5"x ½" tube. This was heated half an hour in a 60°C. water-bath, cooled and centrifuged, the supernatant being discarded. The deposit was then tested in the usual way.

For mannose inhibition testing, 1 loopful of 2% D-mannose in distilled water was mixed with the drop of red cells on the cold tile, before adding the bacterial suspension.

Results:

Haemagglutination results of 28 HILB strains, 1 H.influenzae strain and 1 H.canis strain against 6 species of red cells are given in Table 74. Twenty-two of the HILB strains gave haemagglutination in the cold with 1 or more species of red cell. Of these, 17 gave definite agglutination of human Group O cells, 7 agglutinated horse cells, 10, ox; none, sheep; 1, rabbit; and 11, fowl cells. Three strains, T 182, T 279, and 880

TABLE 74.

Haemagglutination by 30 strains of HILB, using blood of various species.

a.

Strain T number	Species of blood												Amount of growth
	Human		Horse		Ox		Sheep		Rabbit		Fowl		
	Cold	RT	Cold	RT	Cold	RT	Cold	RT	Cold	RT	Cold	RT	
30	-	-	+	-	-	-	-	-	-	-	-	-	+++
34	++	-	±	-	+	-	-	-	-	-	+++	-	+++
38	-	-	-	-	-	-	-	-	-	-	-	-	+++
91	-	-	++	-	-	-	-	-	-	-	+	-	++
100	++	-	+++	-	++	-	±	-	-	-	+++	-	+
109	-	-	-	-	-	-	-	-	-	-	+	-	+++
111	-	-	-	-	-	-	-	-	-	-	-	-	+
131	+++	-	-	-	-	-	±	-	-	-	-	-	+
145	+++	-	-	-	+++	-	-	-	-	-	+	-	+
182	+++	-	-	-	-	-	-	-	-	-	-	-	+
194	-	-	-	-	-	-	-	-	-	-	-	-	++
216	+++	-	-	-	±	-	-	-	-	-	+	-	+
217H	+++	-	-	-	+	-	-	-	-	-	-	-	++
219	++	-	+	-	++	-	-	-	-	-	+	-	++
221	-	-	-	-	-	-	-	-	-	-	-	-	+++
623	-	-	++	-	++	-	±	-	-	-	+	-	+++
693	-	-	+	-	±	-	-	-	-	-	-	-	+++
880	+	-	-	-	-	-	-	-	-	-	-	-	+++
281	-	-	-	-	-	-	-	-	-	-	-	-	+
<u>H.canis</u>	+++	-	-	-	-	-	-	-	+	-	+	-	+

TABLE 74.

b.

Strain T number	Species of blood															
	Human group O			Horse			Ox			Sheep			Rabbit		Fowl	
	Cold			Cold			Cold			Cold						
	1	2	RT	1	2	RT	1	2	RT	1	2	RT	Cold	RT	Cold	RT
46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
92	++	+	-	+++	+++	-	++	++	-	-	-	-	-	-	-	-
94	+++	+++	-	-	-	-	+++	+++	-	-	-	-	-	-	+	-
104	+++	++	-	-	-	-	+++	+++	-	-	-	-	-	-	+	-
115	+++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
119	+++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
170	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
183	++	+	-	-	-	-	+++	+++	-	-	-	-	-	-	-	-
283	+++	+	-	-	-	-	-	-	-	±	-	-	-	-	-	-
279	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Control	-		-	-		-	-		-	-		-	-	-	-	-

1 = original haemagglutination in the cold.

2 = haemagglutination after reversal by warming, and subsequent re-cooling.

RT = room temperature.

+ = very fine granulation, with ring of deposited cells round the drop.

++ = intermediate between + and +++

+++ = coarse granules, developing quickly.

agglutinated human cells only, T 30 agglutinated horse cells only and T 109, fowl cells only. Five strains negative with human cells were positive with one or other of the remaining species, including 4 positive with horse, 1 with ox and 3 with fowl.

All haemagglutinating strains showed reversal of the agglutination when the tile was warmed to room temperature, and all but 1 (T 279) gave agglutination again when re-cooled. Some showed slight diminution of the reaction, after elution and re-agglutination, probably due to the agglutinated cells adhering to the rim of the drop and drying in position during the warming and cooling process. This probably also explains the failure to show re-development of agglutination in T 279, where the original reaction was a weak one. With a weak reaction, indeed, sometimes the only way one could tell that agglutination was present was by the development of a more sharply defined rim of cells round the test mixture than in the control (v Fig. 34).

There was no apparent relation between the amount of visible growth in the culture and the strength of reaction produced, e.g. T 100 gave a poor growth, but a strong reaction with horse and fowl, and moderate with human and ox cells,

whereas T 30, which gave a good growth, only gave a weak reaction with one species of red cell, namely horse. However, T 34, with a heavy growth, also gave a good reaction.

Effect of pH:

This is seen in Table 75.

Table 75. Effect of pH on haemagglutination, using human group O cells.

pH	Strain T 94			Digest broth control	
	Cold		R.T.	Cold	R.T.
	1	2			
3.0				+++	+++
4.0	++	++	-	-	
5.0	+++	+++	-	-	
6.0	+++	+++	-	-	
7.0	+++	+++	-	-	
8.2	++	++	-	-	
9	++	++	-	-	
10	-	-	-	-	

Cold 1 = original reaction. Cold 2 = reaction after warming and cooling. R.T. = room temperature.

Bacterial haemagglutination could be observed at pH levels ranging from 4.0 - 9 but not at 10. Digest broth at pH 3.0 produced acid haemagglutination,

irreversible by warming to room temperature, so that it was not possible to demonstrate bacterial haemagglutination at this pH level. This result compares with that reported by Duguid _et al_.(1955) who state that haemagglutination by _Bact. coli_ occurs over the pH range 3-12.

Effect of formaldehyde:

a. _One per cent_:

Two strains, T 145 and T 94 were used. The deposits, formolised as described above, gave strong agglutination of human Group O cells in the cold, which was reversed by warming to room temperature, and re-appeared on cooling again. A control test using 1% formaldehyde broth with no organisms, gave a negative result in the cold.

b. _Two per cent; after 3 hours at 37°C_.

This abolished the haemagglutinating powers of the 4 strains tested, for human red cells, control deposits incubated for a similar period remaining as strongly positive as before.

Effect of heating:

i. _Boiling_:

This was tried on T 145 only. The boiled suspension failed to haemagglutinate either in the cold or at room temperature.

ii. _60°C. for 30 minutes_:

This also abolished haemagglutination of 3 strains tested.

Effect of mannose (approximately 0.5%):

The results are seen below, the symbols meaning the same as in Table 74.

		Mannose added	Mannose not added
Strain	T 91	+	+
	T 94	+++	+++
	T 145	+++	+++
	T 98	±	±

The mannose therefore had no effect, even the very weak reactions of T 98 and T 91 remaining unaffected.

Haemagglutination-inhibition by antiserum:

The results of adding 5 sera to 2 antigens are seen in Table 76. Haemagglutination-inhibition took place with T 145 and T 94 and a little also with T 62 serum. It was not related to precipitation group, as T 216 failed to inhibit the agglutination of T 145 antigen, which reacts strongly with it in the precipitation test. Similarly, T 46 which reacts with T 94 antigen in the precipitation test, failed to inhibit haemagglutination by that strain. There is insufficient evidence as yet to say whether the anti-haemagglutinins are specific in sera T 145 and T 94. They may be non-specific inhibitors analogous to Francis and normal serum inhibitors of influenza virus haemagglutination.

TABLE 76 .

Haemagglutination inhibition.

Antigen	Antiserum					Control no serum
	T216 Gp.1	T145 Gp.1,2	T46 Gp.2	T94 Gp.2	T62 Gp.3	
T145 Gp. 1,2	+++	-	+++	-	±	+++
T94 Gp. 2	++	-	++	-	+	++

+, ++, +++, - as in Table 74.

Note: T145 serum proved useless in precipitation tests, the group being decided by the reaction of T145 antigen with other sera.

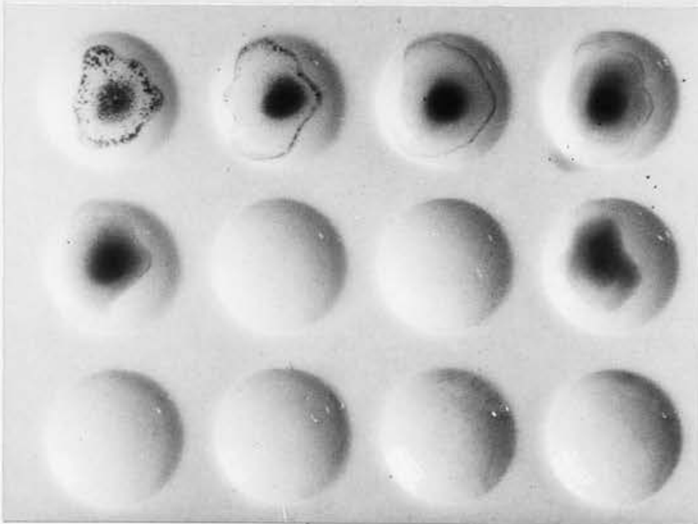


Fig. 34. Cold agglutination of human red cells by strain T145. Deposit diluted 1/1,1/2,1/4,1/8 and 1/16 from L. to R. In the last depression of the 2nd row is the red cell control.

Slightly less than actual size.

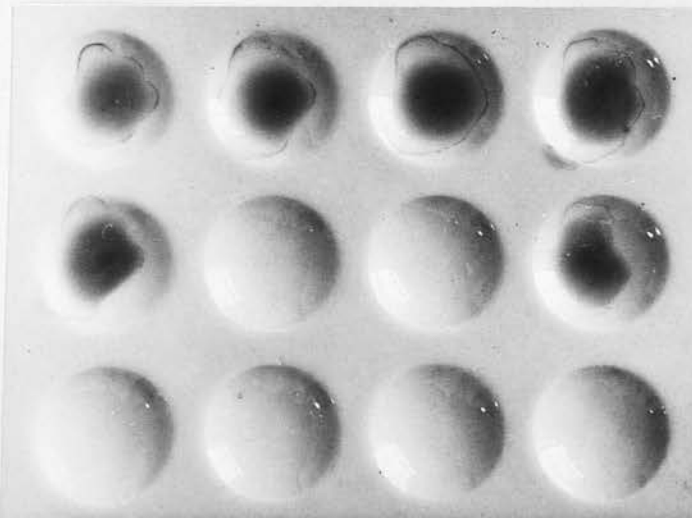


Fig. 35. This is the test shown in Fig. 34 after warming to room temperature, showing reversal of the haemagglutination.

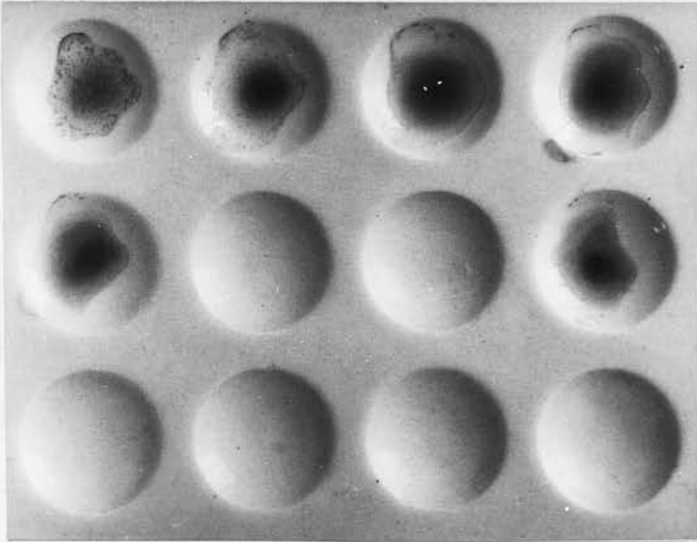


Fig. 36. The same test as in Figs. 34 and 35 after re-cooling, showing haemagglutination slightly less marked than in the original test.

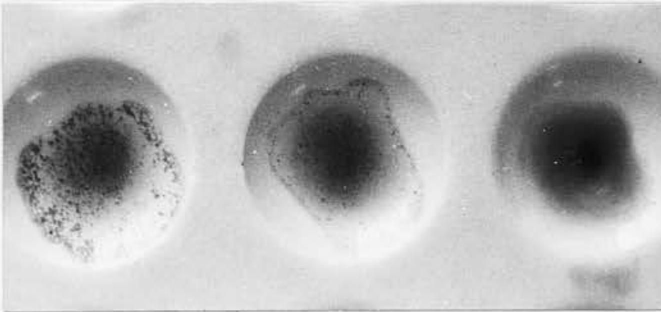


Fig. 37. Haemagglutination by HILB showing a +++, ++ and - reaction, reading from L. to R.

X 1.25

Section 4: Relation of PPLO to HILB:

When work was first started on HILB, the possibility was not appreciated that many strains of PPLO would grow on our routine blood agar. After isolation of HILB by picking single colonies, continued sub-culture showed in some cases an apparent dissociation of the culture into 2 types of colony (1) larger and haemolytic, (2) smaller and non-haemolytic, and in greater numbers than (1). This happened often after repeated sub-culture of what was thought to be a pure culture e.g. the cases of strains 239 and 623. When Gram films were made of the non-haemolytic (NH) variants, only woolly masses with some swollen globules and club-shaped forms could be seen. It was then thought that these forms might be PPLO, and this was proved by microscopic examination of the colonies on soft blood agar plates. A spot-light was trained obliquely on the colony from beneath the plate, the condenser of the microscope being removed. When a sufficiently thin layer of medium was present between the colony and the light source, the low power objective was able to show the typical morphology of PPLO (v Figs. 1,2) with central compact portion surrounded by a halo of more transparent, fuzzy growth. These colonies were penicillin-resistant, in common with all PPLO, and later this

property was used to separate the 2 forms in the case of a strain isolated later.

During the collection of the main series of HILB strains, the possibility of contamination with PPLO was realised, and particular care was taken in picking colonies, when PPLO was known to be present. This was very necessary because of the extreme smallness and transparency of the PPLO colonies, even smaller than HILB and more slow-growing, but in very large numbers. Often repeated plating and picking from single isolated colonies was necessary to obtain a genuinely pure culture of HILB. From then on, no case was ever observed of HILB undergoing transformation to PPLO, or vice versa.

Some strains of HILB showed degenerated forms when films were made of cultures on blood agar (v Chapter III). The outlines of the bacillary walls were faint, and globular and club-shaped swellings were seen at the ends of some of the bacilli. These appearances suggested incipient L-forms, although they were not so obvious as those shown by Amies and Jones (1957) to occur in H. vaginalis under the influence of penicillin.

A few observations were made on the biochemistry of PPLO strains isolated during the investigation. During preliminary work on suitable media for fermentation and other biochemical reactions it

was noted that 2 non-haemolytic strains, later shown to be really PPLO, always failed to ferment carbohydrate, when positive results were being obtained with the other 4 HILB strains being tested. They thus differed from HILB in their lack of fermentative power. Adonite, lactose, glucose and maltose were twice tested in plasma digest broth with the 2 strains of PPLO (575 and 999) and were negative, although growth occurred in every case, as checked by sub-cultures after 2 - 3 days. It was noticed, in fact, that 575 and 999 were easier to maintain in culture, growing on any medium containing blood, plasma or serum even when HILB strains were failing to grow.

Later, 4 PPLO strains isolated along with HILB from high vaginal swabs were tested by method (2), Chapter V, Section 1, growth from thallium acetate plasma agar being emulsified in plasma, and 0.15 ml. of the emulsion being pipetted into 3 ml. sugar medium. Readings were made as for HILB. The sugar media included both 1% and 0.25% concentrations, so that 2 tubes were used for each sugar per strain. The PPLO strains were Tp38 (the"p" indicates that the strain is PPLO) Tp111, Tp115, Tp131. All were negative after 12 days incubation, although numerous positives were being obtained from the same batch of medium, with HILB strains.

Other biochemical reactions were also negative. Thus, strains 575 and 999 were grown successfully in plasma digest broth (PDB) containing urea and phenol red, but failed to show any urease activity. Phosphatase, as tested with PDB + phenolphthalein diphosphate, was not produced, and the two strains, when cultured in glucose phosphate peptone water, with added plasma, grew, but gave a negative Voges-Proskauer and methyl red reaction. PDB cultures were negative for indole, although E. coli gave a good positive reaction in this medium. Nitrate was not reduced when the organisms were grown in nitrate-containing plasma broth. Although a few HILB strains were able to reduce nitrate under ideal conditions, using massive suspensions to avoid the necessity for growth, tests using ordinary inocula gave negative results, so that there is not necessarily a difference here between PPLO and HILB.

Serological reactions of 4 PPLO strains were tested as follows:-

One oz. bottles of 10% plasma digest broth were inoculated with thallium acetate agar cultures of PPLO strains, Tp38, Tp111, Tp115, and Tp131. After 5 days incubation at 37°C., all bottles showed good growth. They were centrifuged and the deposits pooled and extracted by the HCl method in the same way as HILB extracts were made. Precipitation

reactions were carried out with HILB sera. The results are given in Table 77.

Table 77. Cross precipitation between HILB and PPLO:

Antigen	Serum									
	T30	T34	T38	T46	T62	T94	623	T194	T238	T170
Tp38	-	-	-	-	-	-	4	-	-	-
Tp111	-	-	-	1	4	-	8	-	-	-
Tp115	-	±	-	-	4	-	8	-	+	+
Tp131				2		1	8	-	-	+

+ = weak positive with undiluted serum; ± = doubtful positive, - = negative. Titres are expressed as the reciprocal of the serum dilution at the end-point.

623 is the number of an old strain from which an anti-serum was made after a very prolonged period of immunisation. It was not included in the main series, because of the likelihood of non-specific cross-reactions being emphasised by its mode of production. At the time when the course of injections of this strain was started, its dissociation into HILB and PPLO had not been observed, and it now seems likely that it was in fact a mixture of HILB and PPLO at the time. During the first few weeks of the course, therefore, antigen had been injected which in all probability contained many PPLO. This is believed to be the explanation, at

least in part, of the strong reaction given by this serum with PPLO extracts. This explanation does not, however, apply to the other, weaker, reactions given by other sera. T 62 gives a quite definite precipitation with Tp111 and Tp115, which are PPLO isolated from the same specimens as T 111 and T 115 respectively. T 111 and T 115 also react with T 62 serum, being allocated to precipitation group 3. Tp111 and Tp115 are therefore serologically related to each other and to T 111 and T 115.

Attempts to produce PPLO from HILB:

Eight successive sub-cultures of strains, 239, 623, 693 and 880 were made on soft blood agar (New Zealand agar 0.5%) with a 1 unit disk of penicillin added each time. The inoculum for each sub-culture was taken from the region of growth at the edge of the zone of inhibition, so as to include any PPLO forms that might be developing. No growth of PPLO occurred, the HILB strains remaining fully sensitive to penicillin throughout the series.

This method was tried again with 6 HILB strains, and other L-forming agents were included in the experiment.

Glycine is known to stimulate the production of L-forms (Dienes et al., (1950). Sodium chloride and lithium chloride were stated by Klieneberger-Nobel (1949) to have a similar effect. The latter

also described the L-forming effect of cold.

Saturated aqueous solutions of glycine and NaCl, and a 3% solution of LiCl were steam-sterilised and added to a hole bored in soft blood agar, after inoculation to the edge of the hole with HILB strains T 38, T 111, T 131, T 216, T 145 and T 46. The first 4 strains had originally been accompanied by PPLO in the vaginal specimen, the last 2 had not so far as was known. In addition, a 5 unit disk of penicillin was used as described in the previous experiment. All cultures were this time incubated anaerobically, since some PPLO and L-forms grow better in this way (Weinberger et al., 1950). The inoculum was from a 48-hour blood agar plate.

After 48 hours anaerobic incubation at 37°C., zones of inhibition were observed with the above strains in the case of each substance used. Subcultures were taken from the edge of the zone and this process was repeated 5 times with NaCl, glycine and penicillin, 4 times with LiCl. No colonies resembling PPLO were seen in the zones of inhibition at any time.

The effect of cold was tried by placing a 48-hour blood agar culture in the refrigerator and sub-culturing after 1 hour and after 24 hours on to soft blood agar with a penicillin disk added. No

PPLO colonies appeared after incubation for several days.

Effect of anaerobic growth on cholesterol digest agar:

The 2nd. sub-culture of 4 strains cultured anaerobically on cholesterol digest agar (v Appendix no. 65) produced after 3 days at 37°C. a growth which showed the degenerated type of microscopic morphology already alluded to in Chapter III, to an unusually marked extent. Many swollen forms occurred, together with very faint and slender bacilli and some large coarse beaded partly Gram positive forms (v Figs. 38, 39). Sub-cultures were made on soft blood agar and on a further plate of cholesterol agar, with an added penicillin disk in each case, and anaerobic incubation carried out. No PPLO colonies developed on either medium.

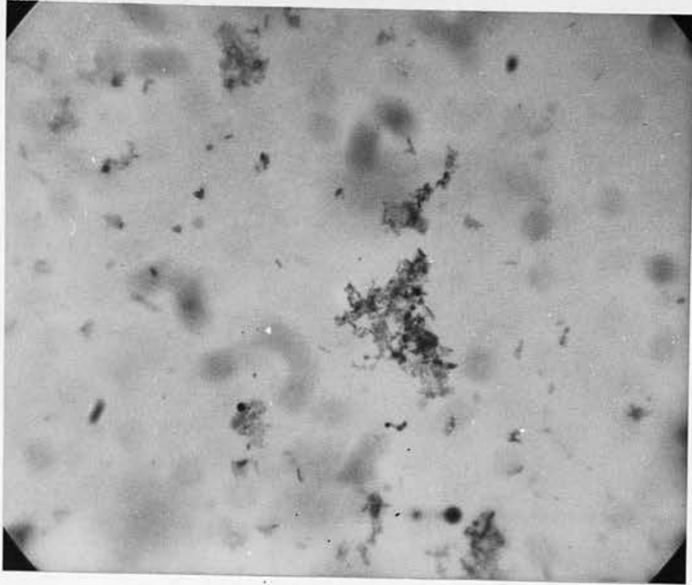


Fig. 38. 3-day anaerobic culture of T145 on cholesterol digest agar, showing ghost forms and swellings.

Stained Gram. X 750.

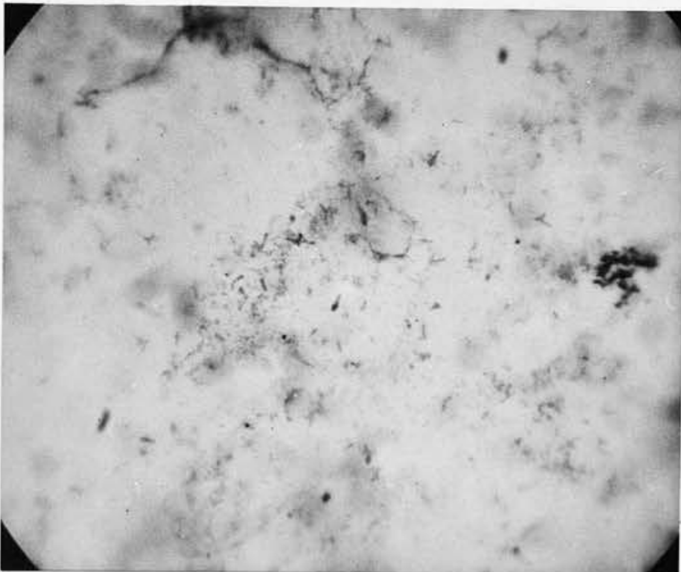


Fig. 39. 3-day anaerobic culture of T30 on cholesterol digest agar, showing ghost forms and swellings.

Stained Gram. X 750.

C H A P T E R VIII.

DISCUSSION, CONCLUSIONS AND SUMMARY.

CHAPTER VIII. DISCUSSION, CONCLUSIONS AND SUMMARY.

Discussion and conclusions:

The factor which initiated the present investigation was the routine use of the blood agar medium developed by Professor W.M. Levinthal and used in this Unit for many years. The details of its production are given in the Appendix, but the important features in so far as HILB are concerned are:-
(1) the use of human blood, which is much superior to horse, ox, sheep or rabbit blood, (2) the addition of horse pancreatic digest made with the minimum possible amount of heating, (3) the use of 2% Evans peptone (with no Lab Lemco) which has also received the minimum amount of heating (4) the use of the minimum amount of agar for adequate solidity (0.8-0.9% New Zealand, according to batch), (5) the use of thick plates (25 ml. in a 4" Petri dish) with the minimum amount of drying and (6) the addition of a small amount of penicillinase to destroy traces of penicillin which the donor may have been given before the blood was collected.

Our finding concerning the superiority of Transfusion Service citrated blood over the blood of other species, contrasts with the use of sheep

blood by Lutz _et al_, (1956) and by Gardner and Dukes (1955) in their studies on similar organisms in France and the U.S.A. respectively. Gardner and Dukes went so far as to state that citrated human blood was unsuitable for solid medium. It may be that their organism, which they call _Haemophilus vaginalis_ is different from HILB, or the medium may differ in other respects, e.g., pH, ordinary laboratory media being near the upper limit for growth of HILB of around pH 7.5, and relatively small increases therefore jeopardising the growth-promoting quality of the medium. It seems likely at this stage of our experience of the organism, that some unexplained failures of growth may have been due to undetected variations in pH, although some cases were adequately explained by the demonstration of traces of penicillin in the medium.

Clinically, the outstanding feature of this organism is, in our experience, its apparent ability to cause puerperal pyrexia. Attempts to arrange for inoculation of volunteers failed, owing to the natural reluctance of clinicians in charge to put at risk patients in the puerperium, so that the evidence for its human pathogenicity is limited to the marked association with pyrexia revealed by comparison with control cases, 71% being the incidence in cases of puerperal pyrexia of 99° F.

or over, as compared with 31% in a control group collected at the same time. The high incidence of positive cultures throughout the series, even in control groups, naturally throws some doubt on the pathogenicity of the organism and it is clear that it does not rank with, for example, Strep. pyogenes as a pathogen of the female genital tract. However, one may draw a comparison here with H. influenzae, which is a commonly occurring organism capable of both commensal and pathogenic properties.

The effects of puerperal HILB infection are not confined entirely to mild degrees of pyrexia, as is seen in Table 3, where, out of 12 cases with a temperature of 100°F. or more, 11 (92%) yielded HILB, of which 9 were in almost pure +++ growth. None of these cases showed evidence of any other possible cause of pyrexia apart from HILB. This power to produce quite severe reactions in a minority of cases has been confirmed by experience since gained, and the unwillingness of the clinicians in charge of the patients to subject them to inoculation with HILB, indicates that they have a considerable respect for its pathogenic powers. In one case, a temperature of 103°F. has been

recorded, without any other cause found than the isolation of HILB in +++ growth.

Previous authors, e.g. Gardner and Dukes (1955), Wurch and Lutz (1955) have stressed the predominance of epithelial cells and the absence or rarity of polymorphs in smears taken from vaginas infected with organisms similar to HILB. We have found, on the contrary, that polymorphs are usually present, both in leukorrhoea and puerperal pyrexia cases, and often in large numbers. Moreover, some of these polymorphs exhibit marked phagocytosis of HILB, in some cases resembling the classical gonococcal or meningococcal picture of intracellular organisms, the diplococci being replaced by small Gram negative bacilli (v. Fig. 7). Recognisable polymorphs showing this phenomenon are rare, but degenerated cytoplasmic masses full of the organisms are often seen, or the picture of Fig. 6 is seen, where there is obvious intracellular packing, but the nature of the cell is not clear. The explanation of this finding is probably that disruption of a polymorph packed in this way is rapid and its debris is more often seen than the intact cell.

Gram variability of HILB:

A remarkable and puzzling feature of the organism is its variable reaction to Gram's stain. Original smears sometimes showed Gram positive forms identical in shape and size to their Gram negative

companions. On culture, however, all the strains were carefully checked for Gram negativity, and any showing Gram positive tendencies were rejected. Later, after repeated sub-cultures, several strains again showed Gram positive tendencies, which were most marked when the organism was grown in liquid medium. The diphtheroid grouping often noticed before was then supplemented by the Gram positive appearance, which made one think it was in fact a diphtheroid bacillus. As already mentioned in the Introduction (Chapter I) two other authors have mentioned this characteristic from their experience of similar organisms. The fact that HILB is not a diphtheroid bacillus is brought out later, in the discussion on the taxonomy of HILB.

Diagnosis of HILB:

If aerobic or anaerobic 37°C. culture of a vaginal or cervical swab is carried out on the blood agar medium described in the Appendix (no.4), the appearance in 48 hours of minute haemolytic transparent smooth colonies, consisting of small slender or pleomorphic Gram negative bacilli, which fail to grow on ordinary medium, but grow well on autoclaved blood agar, indicates the presence of HILB.

The tendency of the bacteriologist, when confronted with the confusing microscopic picture of a high vaginal smear, is to eliminate trichomonas,

yeasts, gonococci and Döderlein's bacilli, and to lump together any other organisms under the term "mixed flora". I suggest that in many cases, these specimens contain in fact an almost pure flora of HILB. This is supported by our extensive experience of cultures from such specimens, where aerobic and anaerobic cultures incubated for 3 days have frequently revealed little other than a copious growth of HILB.

Transport of specimens:

In view of the rapid death of HILB brought about by drying (v. Chapter III, section 5) it is clearly necessary to achieve rapid transport of high vaginal swabs from the patient to the laboratory, or at least to keep the swab moist, e.g., by the use of Stuart's transport medium (Stuart et al., 1954). In any case, the swab should reach the laboratory the same day, as moist broth suspensions die in less than 24 hours at room temperature, (v. Table 17).

Sensitivity to antibiotics and local applications:

HILB are uniformly sensitive to the main antibiotics, particularly penicillin, to which it is even more sensitive than Strep.pyogenes or the Oxford standard staphylococcus. The majority, however, are resistant to sulphonamide and all are resistant to polymyxin. Of 4 preparations likely

to be used for local treatment of vaginal inflammation, nystatin, acetarsol, "pruvagol" (v. Appendix No.70) and gentian violet, only the last inhibited growth. Treatment of vaginal moniliasis with gentian violet will presumably therefore have the advantage over nystatin of dealing with HILB as well as the yeast infection. However, HILB are not so often found in yeast infections as in other vaginal conditions (v. Table 4).

Treatment for HILB infection has so far, in our experience, been limited to puerperal infections associated with pyrexia, and has consisted in the main of administration of systemic penicillin and sulphonamide as a routine for all cases of unexplained puerperal pyrexia. The pyrexia usually has cleared up quickly, but whether this is because of the treatment or because of natural recovery is impossible to say at this stage.

Site of infection:

It seems clear that the uterus is involved in puerperal cases, in view of the pyrexia produced. Uterine involvement is also suggested by the high incidence among cases of cervical erosion, as compared with vaginitis. Thus 7 of 9 HILB positive cases of cervicitis or erosion lacked any of the recognised pathogens, apart from HILB. On the other hand 3 out of 5 vaginitis cases yielded recognised pathogens in addition to HILB. This

contrasts with the findings of Brewer et al, (1957), who thought that "H.vaginalis" was mainly concerned with vaginitis. That their organism could be independent of a uterine nidus was shown by the fact that several patients who had had a complete hysterectomy harboured the organism in the vagina.

Venereal spread:

Leopold (1953) and Gardner and Dukes (1955) showed that H.vaginalis frequently infected the male urethra and that venereal infection could be brought about in either direction, the main reservoir possibly being the male urethra. We did not have the opportunity to explore this interesting aspect as applied to HILB, but we were able to culture HILB several times from the catheter urine of women whose high vaginal specimen yielded the organism. It was not, however, found in urines of women whose high vaginal swabs were negative.

Pathogenicity:

Numerous attempts to demonstrate animal pathogenicity of HILB failed during this investigation, the only suggestive finding being that mild degrees of pyrexia occurred in rabbits inoculated intravenously or intra-peritoneally with large doses of organisms, pyrexia which developed after a few hours and persisted for 4 - 5 days. Guinea-pig

and mouse inoculation and vaginal inoculation of rabbits failed to produce any evidence of illness, or bacterial proliferation. Similarly negative results have been reported by other authors (Lutz *et al*, 1956, Amies and Jones, 1957) the latter, however, stating that a raised lesion was produced by intra-dermal inoculation of a rabbit with a heavy suspension of living "*H.vaginalis*". The other constituents of the injected material were not mentioned. A similar inoculation proved negative in the case of 3 strains of HILB tested by us, using 10% rabbit plasma digest broth as the suspending fluid.

The experiments of Gardner and Dukes (1955) are the only recorded ones in which volunteer women have been inoculated with, in their case, *H.vaginalis*. Thirteen clinic patients, known to be free of infection with the organism, were inoculated with "pure" cultures of *H.vaginalis*. Ten failed to develop either clinical disease or positive cultures. Two yielded positive cultures for 2 and 3 months respectively, but neither developed symptoms. One gave clinical evidence of disease and the organism replaced completely the type I flora previously present. The authors claimed that Koch's postulates had all been fulfilled, but the weak point in their argument was

the rather vague and unsatisfactory criteria used to determine the presence or absence of clinical disease, as already pointed out in Chapter I. Also, their cultures were probably not pure, since they required at least 100 colonies in the inoculum, in order to propagate the organism.

Glycogen fermentation:

In connection with pathogenicity, it is worth considering the possible role of glycogen fermentation. Up till now, the sole cause of the acidity of the normal vagina in the reproductive and neo-natal periods has been recognised to be Döderlein's bacillus, which produces acid by fermentation of glycogen. According to Cruickshank (1934) only Döderlein's bacillus is capable of fermenting this polysaccharide directly, with the production of lactic acid. However, it has been found in this investigation that glycogen is fermented more swiftly and constantly by HILB than any other sugar. Glycogenase occurs in fresh serum (Cruickshank, 1934) and unheated plasma was used in our medium. However, a control tube of 1% glycogen digest broth, containing 5% plasma, failed after incubation for 14 days to give a positive result with Benedict's reagent, showing that any glycogenolysis proceeding independently of HILB must be insignificant. Several strains

giving strong fermentation of glycogen had, moreover, been shown to lack glucose-fermenting powers. Finally, one strain, T145, was able to grow and ferment glycogen in plasma digest broth which had been steamed, to destroy glycogenase. It is clear, therefore that HILB do in fact ferment glycogen. This ability to grow rapidly in glycogen-containing medium indicates a very useful property for establishing itself in the vagina during the reproductive age.

Allied to the glycogen-fermenting ability of HILB is its power of growing well at low pH levels, even down to pH 4.5. Moreover, the viability of the organism was better at low pH levels, possibly because of diminished density of the resulting culture.

Growth requirements:

Chapter IV has shown the complex nature of the growth requirements of HILB. The failure to show satellitism and the ability to grow well on autoclaved blood agar, demonstrated clearly that HILB does not require V factor, since this is destroyed by heating at 120°C., and its absence was confirmed by the failure of H. influenzae to grow on the autoclaved medium unless pure co-enzyme I was added. The same experiment showed incidentally that catalase was not required.

Requirement of X factor has been impossible to prove as yet, any evidence available rather suggesting that it is not the essential factor. Addition of pure haematin solution in varying dilutions has failed to permit growth on any medium where growth did not already occur to an equal or a greater extent in its absence. In particular, the fact that serum digest agar plus haematin, either in 1 in 33,000 or 1 in 1,000,000 concentration, did not support growth of HILB, strongly suggests that the organism does not require X factor, since the medium is on the borderline as regards ability to support aerobic growth of HILB, and has often given good

growth anaerobically. One would think that this normally peroxidase negative medium, would,with added X, be bound to show at least some improvement in growth of HILB if X was in fact required. Further evidence that X-like substances are not important, is seen in the failure of catalase to affect the growth of the organism in media deficient in the substance, e.g. serum digest agar. H.canis on the other hand, which is thought to require X factor (Wilson and Miles, 1955) does show marked improvement in growth when catalase is added to this medium. This is, in fact, the main difference in the growth requirements of the two organisms so far discovered.

H.influenzae, known to require X factor, can grow anaerobically without it, provided the medium is satisfactory in other respects (e.g. presence of V factor). However, if such X-free anaerobic growth occurs, the organism can grow aerobically on the same medium plus X factor in suitable concentration. The relative success of anaerobic culture of HILB, (using cholesterol, lecithin, and other red cell stroma constituents), did suggest a requirement of X factor, since the organism grows well aerobically on blood-containing, and therefore haematin-containing, media. By analogy with H.influenzae, addition of X factor to these media should have

allowed aerobic growth, which, as we have seen, did not occur. Indeed, there was some evidence of actual inhibition of growth by haematin. Also, the strains which produced peroxidase when grown on serum digest agar (the medium itself being peroxidase negative) failed to grow on lecithin digest agar aerobically.

Role of CO_2.

There has been no evidence of a specific requirement for CO_2, but rather that it generally improves growth and viability of cultures.

Role of thioglycollate:

This still remains to be fully elucidated, but the heavy growth obtainable in plain thioglycollate broth is very striking (v. Fig. 26) and growth can also occur on anaerobically (but not aerobically) incubated thioglycollate agar plates. Thioglycollate agar plates give better anaerobic growth than digest agar alone, suggesting that thioglycollate has growth-promoting powers apart from its ability to reduce oxygen tension. The main factors, however, are probably anaerobiosis and the use of a relatively heavy inoculum. Even with the opacity 1/1000 dilution successfully used for inoculating the fluid medium, the number of organisms in the 0.2 ml. of suspension used was still very large, and only a few puff-ball colonies were produced.

Penicillin in transfusion service blood:

An interesting side-line of the investigation was the discovery of traces of penicillin in the transfusion service blood used for culture media. This would not have been noticed if HILB were not so exceedingly sensitive to this antibiotic. It is possible that the cause of defects in growth-promoting properties of certain batches of blood may be the presence of other antibiotics.

Biochemical reactions:

It is difficult to know how much reliance to place on these results, in view of the difficulty or impossibility of growth under the conditions normally stipulated for the various tests. In many cases, there was doubt whether actual growth was taking place, or whether the heavy inoculum was merely lying dormant. In view of the failure to get a positive methyl red test by use of HILB culture media, in spite of the fact that HILB could produce much acid and could survive in acid conditions, an attempt was made to do the test with a really massive inoculum, which was relatively free of the protein of the culture medium. A dense suspension of washed organisms in glucose phosphate peptone water was, in fact, able to give a positive methyl red test in 2 out of 4 strains tested. Voges-Proskauer was negative by this

technique but nitrates were reduced slightly by 3 strains and markedly by the 4th.

Urease and indole tests, using plasma-containing media, were negative, and phosphatase tests gave doubtful or variable results. All 16 strains tested for catalase-production were negative when good growths on serum agar were used, positive controls giving a good reaction. Four of these strains were peroxidase positive, the remainder being negative.

Sugar fermentation was the most useful biochemical test, HILB being found to possess considerable powers in this direction. The sugars fermented included glycogen, starch, arabinose, xylose, maltose, dextrin, galactose, laevulose, and glucose, in descending order of frequency. Lactose, sucrose, mannite, dulcite and glycerol were never fermented. It is surprising that glucose should be the least often fermented sugar (apart from the completely negative ones), as in most organisms this is the most likely of all sugars to be fermented. Moreover, the two most frequently fermented (starch and glycogen)are both multiples of the glucose molecule.

Comparison with other haemophilic bacilli shows the difference biochemically between them and HILB. Stillman and Bourn (1920), found that both

haemolytic and non-haemolytic H. influenzae strains fermented sucrose. This was confirmed by Fildes (1924) for haemolytic strains only, by Dible (1924) for haemolytic and atypical non-haemolytic strains, and by Smith (1931). Rivers (1922) found that H. canis fermented sucrose and mannite and this was confirmed by Fildes (1924). Stillman and Bourn (1920) found that H. pertussis failed to ferment any sugar.

The biochemical reactions of these organisms are summed up by Wilson and Miles (1955) in the following composite table:-

	G	Laev	Gal	Mose	L	S	M	Dextrin	Indole	Nitrate reduction
H.influenzae	+	+	+	+	−	−+	−	−+	+−	+
H.parainfluenzae	+	+−	−+	+	−	+	−	+−	−+	+
H.canis	+	+	+	−	−	+	+	−	+	+
H.pertussis	−	−	−	−	−	−	−	−	−	−

G = glucose; L = lactose; M = mannite; S = sucrose;
Laev = laevulose; Gal = galactose; Mose = maltose

The fermentation of sucrose or mannite, the production of indole, or failure to ferment sugars at all, between them serve to distinguish these organisms clearly from HILB.

Serology:

No previous author has reported on the precipitation and complement-fixation reactions of HILB, but Amies and Jones (1957) described agglutination tests carried out with 25 strains of "H. vaginalis", 18 being agglutinated by one serum and five others agglutinated by a second. The two remaining strains were agglutinated only by their homologous sera. There was no cross-agglutination with 26 H. influenzae and 2 H. pertussis strains tested with these sera. We found no precipitation with one vaginal H. influenzae strain and one H. canis strain by any of the sera prepared against HILB and no complement-fixation with the H. influenzae nor H. pertussis (1 strain). The antigen prepared from H. canis was anti-complementary.

Complement-fixation tests proved unsuitable for serological analysis, using a partly disintegrated suspension of organisms, but further work will have to be done to develop this and other equally sensitive techniques for use in testing patient's serum against strains of HILB isolated from them. The failure of precipitation tests

to reveal any antibody for HILB in the sera of patients infected with this organism may have been due to the relatively insensitive nature of this test.

Haemagglutination:

Many strains of HILB were shown to agglutinate red cells, mainly human, but also horse, ox and fowl in many cases. The haemagglutination only occurred in the cold (with the tile resting on ice), warming to room temperature causing a reversal of the agglutination. Re-cooling the mixture allowed the haemagglutination to occur again. This type of eluting haemagglutinin occurs also in certain haemolytic strains of E. coli (Duguid et al., 1955). Duguid (1959) has shown that this haemagglutinin is independent of the fimbriae described by him and others (1955), that it is resistant to small concentrations of mannose, destroyed by contact with 2% formaldehyde for 3 hours at 37°C. and by heating at 60°C. for 30 minutes. HILB haemagglutinin shows all these characteristics and is therefore not fimbrial in nature, since fimbrial haemagglutination reacts in the opposite way in the above tests.

This type of non-fimbriate eluting haemagglutinin is probably enzymatic in character (Duguid, 1959) in spite of the wide pH range over which its activity is exhibited. The agglutination is probably due to the enzyme in the HILB surface meeting and adhering to its substrate on the red cell, quite independently of any actual chemical reaction, which would be subject to the limitations of enzymatic reaction, and which will in any case be virtually at a standstill at the low temperature at which the haemagglutination occurs. Raising the temperature presumably causes elution of the enzyme to occur too rapidly for HILB to continue to act as a link between adjacent red cells. One may speculate as to the role which this property may have in infection. HILB do in fact adhere to epithelial cells, as in the "clue cell" phenomenon of Gardner and Dukes (1955), and in doing so seem to damage the cell, possibly because of the enzymatic reaction already referred to, which may weaken the cell wall and so allow leakage of its contents to occur. This may be of nutritional benefit to the adhering organism (Duguid, 1959).

<u>Comparison with similar organisms described by other authors:</u>

All the authors shown in Table 78 are agreed in describing their organisms as small Gram negative

pleomorphic bacilli requiring complex media for aerobic growth and very difficult to maintain and purify, which are found in the female genital tract. Positive fermentation results obtained by these authors include fermentation of glucose, maltose, dextrin, galactose, laevulose, xylose and arabinose.

The main differences in the various descriptions are given in Table 78.

Amies and Jones (1957) described the rough character of suspensions made from apparently smooth colonies, just as we found them, but several features seem to differentiate their organism from HILB, especially fermentation of sucrose and glycerol, which we found uniformly negative, and the large size of colony obtained by them (0.5 - 1.0 mm. in 24 hours). Amies and Jones mentioned the possibility of confusing it with a colony of a faecal streptococcus, which would be quite out of the question in our case, the difference in size being so great at a given time of incubation. Another marked difference was the inability of Amies and Jones' organisms to grow under completely anaerobic conditions. HILB grow slightly better anaerobically than aerobically, and indeed anaerobiosis enables it to grow under conditions in which aerobic growth cannot occur, e.g. on cholesterol digest agar.

Although Amies and Jones stated that "H.vaginalis" was not the same as Leopold's (1953) organism,

TABLE 78.

Comparison of HILB with similar organisms described by other authors.

Authors	glycerol fermentation	sucrose fermentation.	Haemolysis on blood agar: blood species not given	human citrated	sheep	rabbit	growth in: plain thio-glycollate	serum or plasma thioglycollate	Colony size on soli[d] medium: 24 hours	48 hours	fermentation in cystine trypticase agar	Clinical source: vaginitis	cervicitis	male urine	puerperal pyrexia	Gram variability	nitrate reduction	penicillin, sensitivity	growth on anaerobic blood agar	over all incidence (female only).
Leopold (1953) (Haemophilus)	-	-	+				+			pp	+		+	+			-			27.4%
Lutz et al (1956) (H.haemolyticus vaginalis).		v			+ G					vs						+	-	+		
Gardner & Dykes (1955) H.vaginalis.	-	-		ns	v		+ pb ma			pp	+	+						-		12.1%
Amies & Jones (1957). H.vaginalis.	+ 12/23	+ 10/23	NG					+ A only	0.5 -1mm.				+				+	±	-	5.1%
Brewer et al. (1957) H.vaginalis.						-	+ mx			dd pp		+				+				
Edmunds (1959). HILB.	-	-		+	-	±	pb+ A,AN	+ A, AN		pp	-	+	+		+	+	+	+	+	39%

dd = dew drop
mx = mixed (not pure)
v = variable
G = greening present

ma = micro-aerophilic
NG = no growth
A = aerobic
AN = anaerobic

ns = not suitable
pp = pin-point
vs = very small
pb = puff-balls

because the latter was "haemolytic, definitely micro-aerophilic, grows in plain thioglycollate broth and does not reduce nitrates", this description is actually a nearer approach to Gardner and Dukes' "H.vaginalis" (which Amies and Jones assumed to be the same as their organism) than Amies and Jones' organism seems to be. Gardner and Dukes described the same micro-aerophilic growth on plain thioglycollate broth (minus eH indicator) as Leopold did, and they mention haemolysis as a variable feature which aided recognition when present, in common with Leopold, whose organism was haemolytic. On the other hand, Amies and Jones' organism would not grow in plain thioglycollate medium and when growing with serum added grew only aerobically. Finally Amies and Jones made no mention of haemolysis, presumably because their organism failed completely to grow on blood agar.

The main difference between HILB and the organisms of Leopold, and Gardner and Dukes, is that HILB has so far failed to grow in cysteine trypticase agar. In view of recent successful efforts in growing HILB in plain thioglycollate broth (not Oxoid fluid thioglycollate, which is inhibitory) and on anaerobic blood-free solid media,

there seems no fundamental difference in growth requirements between this organism and that of Gardner and Dukes.

However, HILB is uniformly sensitive to penicillin, whereas "H.vaginalis",as reported by Gardner and Dukes, is resistant. This finding may well be due to admixture with PPLO, since these authors were unable to culture single colonies, the inoculum required being about 100 of these. We have found that it is difficult to separate HILB from PPLO even when single colonies are picked, so it seems that the chance of Gardner and Dukes' organism being contaminated with PPLO is high.

The above authors reported varying incidence rates for their organisms. Thus, Gardner and Dukes (1955) found positive cultures of "H.vaginalis" in 141 of 1181 patients, a total incidence of 12%. Amies and Jones (1957) obtained cultures of a similar organism from only 5.1% of 371 cervical swabs. Leopold (1953) successfully isolated his strain from 27.4% of 58 cervical swabs. These figures compare with the overall incidence of 38.9% of 276 cases found in the present series, including 71% of puerperal pyrexia cases and 43% of gynaecological, a considerable percentage also occurring in the control group, e.g. 31% in puerperal controls, 27% in ante-natal normals and 21% in gynaecological controls.

Relation of HILB to PPLO.

The association occurring between HILB and PPLO has already been pointed out (v. Chapter II), there being a significantly higher proportion of PPLO isolations from HILB positive cases than from negative. It has also been shown that this is not due to a common association of the 2 organisms with the higher pH ranges. This common association does exist, but does not explain the fact that PPLO isolations are also more frequent among HILB positive cases of pH < 5.4 than among HILB negative cases of the same pH.

Some difficulty was experienced on several occasions in ridding HILB cultures of PPLO. Single colonies were invariably picked from blood agar at first isolation, but it was found that, after a few serial sub-cultures, a non-haemolytic variant began to appear and to outnumber the haemolytic HILB colonies. These variants were then shown to be in fact PPLO. This suggested that the PPLO might have arisen from the HILB.

Another phenomenon suggesting a relationship between PPLO and HILB is the occurrence of degenerated markedly pleomorphic swollen forms of HILB under certain conditions, e.g. anaerobic culture on cholesterol digest agar. These looked like L-forms. Amies and Jones (1957) have already described the production of L-forms from "H.vaginalis" under the

influence of penicillin, and deduce from their findings that PPLO and HILB may be two morphological forms of the same organism. Certainly, the relation of PPLO and HILB to uro-genital disease seems to be very similar, both being present in a large percentage of normal people and yet showing a higher incidence in local disease than in normal specimens, both on occasion giving rise to quite serious and sharp reactions. For example, Randall _et al_, (1950) found a higher incidence in cases of leukorrhoea (55% in moderate and 71% in severe cases) than in normal (overall incidence in cervices of 300 women, 26%). In one case, PPLO was the only organism isolated from aseptically collected pus from a tubo-ovarian abscess. (This does not fit very well with the argument of Amies and Jones that PPLO are non-virulent and "_H.vaginalis_" the virulent bacillary form of PPLO). Similarly, HILB is more frequently involved in the more severe puerperal pyrexias than in the mild forms (v. Chapter II), and in one case has been the only likely causative organism found in a case with a pyrexia of 103° F.

Common relation to diphtheroid bacilli:

Several authors have now described the formation of diphtheroid variants from apparently pure cultures of PPLO, e.g. Minck (1953), Peoples _et al_,

(1955), Smith et al,(1957) and Wittler et al,(1956). This occurred in liquid cultures, the PPLO predominating on solid medium. The "derived" diphtheroids failed to ferment carbohydrates (Smith et al, 1957) and showed cross-agglutination with the parent PPLO strain but not with non-diphtheroid-producing PPLO strains. Attempts at conversion of these diphtheroids back to PPLO were unsuccessful.

HILB also show diphtheroid tendencies at times, Gram positive granular or beaded forms arising, again in fluid cultures. These are quite pure, sub-culture on blood agar showing only the typical haemolytic Gram negative HILB forms. So one has the relationships HILB - diphtheroid, PPLO - diphtheroid, HILB - L-forms (resembling PPLO).

Other common features between HILB and PPLO include the extremely small size of colony of HILB, some of which may be non-haemolytic, and which produce pitting in the surface of the agar; the slow growth; the requirement of large amounts of serum if blood is not used; the preference for soft moist agar; similar atmospheric requirements; biochemical inertness (apart from carbohydrate fermentation); the similarity of the list of sugars fermented by HILB and those strains of PPLO that do ferment carbohydrate (v. Edward, 1954) including starch and glycogen; the occurrence of cross-pre-

cipitation between them; and the requirement for cholesterol of HILB, when grown anaerobically without blood or serum. Edward (1953 and 1954) showed that PPLO required this substance when serum was replaced by albumin plus the acetone-insoluble fraction of egg-yolk.

The differentiating factors between the two organisms include the following:-

HILB are bacilli with definite morphological features and readily stained by ordinary techniques. Colonies do not show the features of PPLO, being definitely haemolytic, having no central and peripheral zones and not being embedded in the agar as PPLO are. After numerous sub-cultures, no transformation of pure cultures from HILB to PPLO or vice versa has been observed. The apparent dissociation noted above shortly after isolation of HILB cultures, occurred only when PPLO were known to have been present in the original specimen or when no steps had been taken to ascertain their absence. Extra care in picking technique, after the danger of contamination with PPLO had been appreciated, eliminated the occurrence of dissociation in later experience.

Repeated efforts to produce PPLO from HILB have consistently failed, using L-form-stimulating substances such as penicillin, glycine, lithium chloride, strong sodium chloride and the effect of

cold.

PPLO are prevented from growing by the presence of antibody and complement, unlike bacteria (Edward, 1954). During an attempt to demonstrate anti-haemolysin in HILB immune serum, undiluted fresh serum was applied to the surface of a blood agar plate and HILB inoculated over it. Growth was unimpaired.

Biochemically HILB were quite different from the PPLO strains accompanying them in the specimens and at one time thought to have been derived from them. Six such PPLO strains were tested for fermentation of sugars and consistently failed to give any result, although growth occurred and the indicator (bromthymol blue) would have shown the slight change in pH characteristic of PPLO fermentation.

In spite of the many resemblances and relationships existing between HILB and PPLO therefore, one can only conclude that no evidence yet exists to contradict the view of Klieneberger-Nobel (1954) that PPLO had not been shown to be derived from bacteria and that the group was to be regarded as a distinct class of micro-organisms intermediate between bacteria and viruses.

Attempts to correlate various properties of different strains of HILB:

An attempt was made to use the various properties of HILB in order to form sub-groups of the

organism. Lists were made of all the strains showing similar characters, including morphology, cultural, haemolytic, haemagglutinating and serological characters, relation to PPLO and clinical groupings. For example, all those strains originally showing a predominance of cocco-bacillary forms in culture were listed separately from those showing mainly diphtheroid types of grouping, and from those showing metachromatic granules on maltose blood agar. Those showing stimulation of growth by CO_2, those fermenting 4 or more sugars and 2 or less were listed separately. The peroxidase positive and negative strains, serological and clinical groups were listed also. The lists were then compared to see if strains kept appearing together in the same group.

The result was a failure to discover any correlation between the occurrence of the various characters. Thus, of 8 strains showing predominantly cocco-bacillary morphology, 2 were from the puerperal pyrexia group, 2 from puerperal control, 2 from ante-natal, 1 from post-menopausal and 1 from gynaecology control groups. The same lack of association between any two characters occurred throughout. In particular, serological grouping by precipitation tests showed a haphazard distribution of clinical groupings within each precipitation group, with one exception, namely, no PP

strains occurred among the 5 strains belonging to precipitation group 3, although the number of PP strains among 52 strains tested was 25, i.e. half. All the other precipitation groups of any size had about half of their number from the PP group.

HILB seem in fact remarkably homogeneous in their characters, many of the variations observed being themselves variable (e.g. morphology and sugar fermentation) and sub-groups such as the serological and clinical ones being in the main independent of each other. It may be that later work on growth factors may enable more reliable and consistent results to be obtained with biochemical reactions and thus give grounds for sub-divisions of epidemiological importance.

Taxonomy:

As the name "H. influenzae-like bacillus" implies, the most obvious genus for the organism would be Haemophilus. It requires blood or its constituents for aerobic growth. However, especially in view of the doubt as regards its X factor requirement, and its lack of V-factor requirement, other possible genera will now be surveyed, if only to dismiss them after due comparisons have been made.

Starting with the definition "small Gram negative, aerobic and facultatively anaerobic, non-motile bacillus, requiring complex media for growth", and following the natural keys in Bergey's Manual of Determinative Bacteriology (Breed et al., 1957) the organism is quickly assigned to the class Schizomycetes, order Eubacteriales, family Brucellaceae. However, the tendency to formation of degenerated forms, and the relation apparently existing between HILB and PPLO, suggests *Streptobacillus moniliformis*, (Levaditi* et al., 1925) so that this organism will also be considered. In addition, the spatial arrangement of the bacilli in films of cultures, together with their Gram variability, suggest diphtheroid bacilli and the genus Corynebacterium is therefore included in the discussion.

* Full references for the names and dates mentioned after each species in the Taxonomy section can be found in Bergey's Manual (Breed et al., 1957).

According to Breed et al., (1957), Streptobacillus is the only member of the family Bacteroidaceae which is not strictly anaerobic. It resembles HILB in its growth requirements, sugar fermentation reactions, Gram variability and its ready production of L-phase forms. It is readily distinguished, however, by its pathogenicity for mice, the spontaneous formation of moniliform bodies and filaments up to 100μ in length, and the large size of its colonies on serum agar (1.0 - 2.5 mm. in 3 days, compared with 0.3 mm. of HILB).

Corynebacteria may show Gram negative forms in young or old cultures and the angled arrangement typical of their dividing cells is seen also in HILB cultures. They produce rough suspensions and great difficulty is encountered, as in HILB, in performing agglutination reactions. All except C.pyogenes (Glage, 1903) grow on ordinary media, however, and, according to Bergey, all are catalase positive. They are therefore quite different from HILB, which require blood or serum, and are catalase negative. The haemophilic Gram positive bacilli described by Svendsen (1947) were said to require X and V factors, but they were strict aerobes and definitely Gram positive, being unlike HILB in both respects. Stein (1948) described satellitism of a

corynebacterium, due to a filterable growth factor produced by staphylococci, of protein nature, probably albumin, and not X or V or any of the known factors promoting growth of corynebacteria. If truly haemophilic bacteria are to be assigned to the genus Corynebacterium, then HILB would have to be considered as a possible candidate for this genus, but in the meantime they must be excluded from it.

The family Brucellaceae includes the following genera, according to Breed et al., (1957):- Pasteurella, Bordetella, Brucella, Haemophilus, Actinobacillus, Calymmatobacterium, Moraxella and Noguchia. Most of these can be eliminated as follows:-

Pasteurella:

The only species not growing on ordinary media are Past.tularensis (McCoy and Chapin, 1912) and Past. novicida (Larson et al., 1955). The latter ferments sucrose, is pathogenic for laboratory animals and gives much larger colonies than HILB, even on yeast extract agar, which fails to support the growth of HILB. The former is strictly aerobic, extremely pathogenic to a wide variety of animals and causes tularaemia in man. It differs in all these properties from HILB.

Bordetella:

The only member of this genus at all resembling

HILB is Bordetella (or Haemophilus) pertussis (Holland, 1920). Apart from its pathogenicity, this organism differs in many respects from HILB. It loses its original strict growth requirements easily on sub-culture and changes its serological phase from smooth to rough, unlike HILB which remains unchanged in colonial appearance and growth requirements through numerous sub-cultures. H.pertussis fails completely to ferment carbohydrates, as against the usual ability of HILB to do this. It has recently been shown (Brumfitt, 1959) that H.pertussis has essentially the same requirements of X and V as H.influenzae, another point of differentiation from HILB, which does not require V factor.

Brucella:

All 3 species grow on ordinary agar, are purely cocco-bacillary and have different pathogenic properties from HILB. They need only to be mentioned to be dismissed.

Actinobacillus:

The members of this genus all grow on ordinary media except Actinobacillus actinoides (Smith, 1918) which produces clubs and sheathed filaments quite unlike HILB. Its source, from pneumonia of calves, also differentiates it from HILB.

Calymmatobacterium granulomatis (Donovan bodies):

This organism is included by Breed et al.,

(1957) in this family. It fails to grow on inanimate media, however, until after adaptation from yolk sac or amniotic fluid culture. This, together with its causation of granuloma inguinale, distinguishes it from HILB.

Moraxella:

These species are actively proteolytic, liquefying inspissated serum or gelatin. They occur mainly as diplobacilli and are especially associated with eye lesions. They differ from HILB in all these properties.

Noguchia:

This genus occurs in the conjunctiva of men and animals, its members are encapsulated and motile and can grow on ordinary media.

There remains the genus Haemophilus to be considered.

Breed et al., (1957) define this genus as follows:-

"Minute rod-shaped cells which are sometimes thread-forming and pleomorphic. Non-motile. Gram negative. Strict parasites, growing only in the presence of certain growth accessory substances. May or may not be pathogenic. Found in various lesions and secretions, as well as in normal respiratory tracts of vertebrates".

This description fits HILB, although V factor is not required and the requirement of X is in doubt.

Wilson and Miles (1955) do not insist on requirement of either X or V for an organism to be included in the genus Haemophilus. The actual name "Haemophilus" does not in any case mean "requiring blood" but "loving blood". HILB can certainly therefore be included in the genus, even if further research shows that X factor is definitely not required.

The lack of requirement of V factor differentiates HILB from H.influenzae (Lehmann and Neumann, 1896) (and the Koch-Weeks bacillus), H.suis (Hauduroy et al., 1937), H.haemolyticus (Bergey et al., 1923), H.gallinarum (Delaplane et al., 1934), H.para-influenzae (Rivers, 1922), and H.parahaemolyticus (Pittman, 1953). H.piscium (Snieszko et al., 1950) affects cold-blooded animals and so can at once be excluded. H.citreus (Diernhofer, 1949) can also be dismissed as it produces a lemon-yellow pigment on blood agar. H.putoriorum (Hauduroy et al., 1937)isolated from the respiratory tract of ferrets, is different from HILB, not only in its source, but in the satellitism it exhibits round other bacteria on ordinary medium. HILB fails to grow on ordinary media even in the neighbourhood of staphylococci or other bacteria.

H.aphrophilus (Khairat, 1940) on 24-hour blood agar cultures, gives much larger colonies than HILB and produces an olive-green discolouration after 3

days. It ferments lactose, and is therefore different from HILB.

H.influenzae-murium (Kairies and Schwartzer, 1936) gives large colonies on Levinthal agar, resembling those of H.influenzae, i.e. quite unlike HILB. Acid is produced from lactose and sucrose. It causes conjunctivitis and respiratory infection in mice. It is in these respects different from HILB.

H.ovis (Mitchell, 1925) grows on ordinary agar after serial passage on chocolate agar. No haemolysis is produced on blood agar. It ferments sucrose, mannite and lactose and differs therefore from HILB.

H.ducreyi (Neveu-Lemaire, 1921) is said to require X factor, but occurs in chains and causes soft sore (chancroid). It otherwise resembles HILB in producing a small, haemolytic colony on blood agar.

H.haemoglobinophilus (Lehmann and Neumann, 1907) (H.canis) is thought to require X factor. It has behaved in our experience very similarly to HILB with respect to X-containing media. The strain used (National Collection of Type Cultures, 8540) did not in fact grow on serum medium with haematin added, but did grow when catalase was used instead. HILB did not grow on serum agar plus

catalase. Other differences between H. canis and HILB included fermentation by the former of mannite and lactose, its source (preputial secretions of dogs), production of indole, lack of haemolysis in blood agar cultures, and, in our experience, the very much larger colonies produced on this medium.

HILB therefore differ markedly from all the above organisms and is entitled to a separate species name. That of Haemophilus vaginalis given by Gardner and Dukes (1955) has precedence and seems valid, provided that their organism is the same as HILB. The two organisms have already been compared, through the literature, earlier in this discussion and the differences seem unimportant, when allowance is made for the fact that Gardner and Dukes were unable to pick single colonies and their cultures were therefore probably contaminated with PPLO (v. also Chapter VII, Section 4). The name Haemophilus vaginalis should therefore be applied to HILB from now on. On the other hand, it seems doubtful if Amies and Jones' (1957) organism is the same, judging by their description, and it should probably not be called H. vaginalis until more is known about it.

Summary:

A description is presented of small Gram negative bacilli resembling H. influenzae, which have been found in 38.9% of high vaginal swabs of 231 women. This organism has up till now been called the H. influenzae-like bacillus (abbreviated to HILB throughout the thesis).

HILB was isolated in pure culture from 71% of 45 cases of mild or moderate puerperal pyrexia with no other known cause, but from only 31% of 26 puerperal control cases without pyrexia. The incidence in 42 gynaecological cases (women of child-bearing age) suffering from leukorrhoea, vaginitis and/or cervicitis, was 43%, but only 21% in a control series of 42 women without these conditions. In both instances a statistically significant positive association was established between the isolation of HILB and the presence of the disease processes mentioned above. The organism was also isolated in a higher percentage of post-menopausal women suffering from leukorrhoea than in control women of the same group. Its incidence in 44 normal pregnant women attending an ante-natal clinic was 27%.

A study was made of the morphological and cultural characters of HILB, its growth requirements, biochemical and serological properties, its animal pathogenicity and its relationship to

pleuropneumonia-like organisms, with which it was found to be associated in high vaginal specimens.

HILB are small pleomorphic Gram negative bacilli, showing a tendency to diphtheroid arrangement and the production of L-forms. Gram positive granules are sometimes produced and some strains become Gram positive in fluid cultures. It is aerobic and facultatively anaerobic, showing a slight preference for added CO_2 in the atmosphere. It is extremely fastidious and difficult to grow, purify and maintain in culture. It requires blood or its constituents for aerobic growth, though anaerobic growth can occur in their absence when fairly heavy inocula are used. It produces minute haemolytic colonies on blood digest agar, reaching only 0.3 - 0.5 mm. in diameter after 3 days incubation at $37^{o}C$. It does not require V factor or catalase, and probably does not require X factor. Red cell stromata are the main source of the growth factors found in blood. Stroma constituents, including cholesterol, cephalin and nucleic acid improve anaerobic growth when added to digest agar, and cholesterol enables this to be maintained in sub-culture for a limited time.

Sugar fermentation is variable, the most frequently fermented substances being glycogen and starch. Lactose, saccharose, dulcite, mannite and

glycerol are not fermented. By virtue of its glycogen-fermenting ability and its resistance to acid, the organism is well adapted to survive in both pathological and normal vaginal secretions.

The majority of the strains tested serologically have been assigned by precipitation reactions to one or other of 9 groups. There is no correlation between clinical source and any particular serological group, except that group 3 does not contain any strain isolated from a case of puerperal pyrexia.

HILB show haemagglutination of the eluting, mannose-resistant, heat- and formaldehyde-sensitive type, and can cause haemolysis of human red cells in tubes and in blood agar plates.

Animal pathogenicity is weak or absent, the only positive finding being a mild pyrexia caused for several days in rabbits after intra-peritoneal or intra-venous inoculation of large doses of living HILB.

The organism should be assigned to the genus Haemophilus and the name "H. vaginalis" already given to a similar organism by Gardner and Dukes (1955) should now be applied to HILB.

A P P E N D I X.

DETAILS OF CULTURE MEDIA, TECHNIQUE OF FREEZE-DRYING AND EXAMPLES OF STATISTICAL CALCULATIONS.

APPENDIX

DETAILS OF CULTURE MEDIA, TECHNIQUE OF FREEZE-DRYING, AND EXAMPLES OF STATISTICAL CALCULATIONS.

A. Culture Media

1. The basic medium used for most of the work was called ordinary broth, or 2% peptone broth. This was made up as follows:-

Evans peptone	20 g.
NaCl	2.5 g.
Na glycerophosphate	2 g.
2N Na_2CO_3	11 ml.
Distilled Water	1000 ml.

Steam 1 hour by free-steaming in autoclave (at 2-5 p.s.i.). Cool and adjust pH to 7.3 if necessary. When using Difco proteose peptone (as in comparison with Evans) only 2 ml. 2N Na_2CO_3 is added instead of 11 ml.

2. Ordinary agar (2% peptone agar) is the above, with 0.8 - 0.9% Davis New Zealand agar, depending on the batch of agar. The agar is soaked overnight in the broth. The steaming is repeated as above to melt and sterilise the agar. It is then dispensed aseptically into 300 ml. sterile bottles (medical flat) and steamed for 20 minutes.

3. Nutrient agar, as used for making lysed blood

plates (Harper and Cawston, 1945, modified by using saponin instead of water for laking the blood, and using 10% extract instead of 5%).

Evans peptone	10 g.
NaCl	5 g.
Lab Lemco	10 g.
2N Na_2CO_3	11 ml.
Distilled water	1000 ml.

Steam and pH as in previous media. Agar is added, melted and sterilised as above.

To make lysed blood plates, add 2.5 ml. of 10% saponin in water to 100 ml. oxalated horse blood and place in 56°C. water bath for 5 minutes (in a 100 ml. medical flat bottle). The lysed blood thus produced is added in 10% concentration to 1 litre of melted and cooled to 50°C. nutrient agar, and plates poured.

Media containing whole blood.

4. Routine blood agar (5% digest blood agar):

To 300 ml. ordinary agar (melted and cooled to 50°C.) is added 15 ml. horse digest, made as described below, and 18 ml. human group O transfusion service blood, which has been rejected as unfit for transfusion because of either age, or a non-infective illness of the donor. This blood is citrated by the transfusion service with the following solution:-

Disodium citrate		2.5 g.
Dextrose		3 g.
Distilled water	to	120 ml.

400 ml. of blood is added.

After the addition of the blood to the melted agar, penicillinase 0.045 ml. is added (1 ml. inactivates 100,000 units penicillin) and thoroughly mixed before pouring plates (20 - 25 ml. in a 4" Petri dish). Drying is carried out as follows, when practicable. After setting, the plates are placed face down, with lids removed and stacked separately, in a 45°C. incubator or even-heat oven at 45°C., and the minimum time allowed for removal of all free fluid from the surface of the medium. They are then assembled and stored in piles of not more than 4 - 5, as it was found that deeper piles gave excessive condensation on the lids of the bottom plates, on storage at room temperature. If, despite these precautions, moisture was found to be still present on the rim of the plate, this was dried off with a piece of sterile filter paper before using the plate. The refrigerator was not used for storage, as excessive condensation was thus caused.

5. Horse digest, method according to Levinthal (1931):

Minced fat-free fresh horse flesh	1000 g.
Distilled water	3000 ml.

2N Na_2CO_3	50 ml.
Pancreatin (BDH)	5 g.
Chloroform	35 ml.

The pancreatin is ground up in 100 ml. of the water and then added to the rest of the ingredients. The mixture is then incubated for 19 hours at 37°C. with intermittent shaking. About 50 ml. of the mixture is removed, boiled till the meat coagulates, and filtered through Whatman's no. 1 filter paper.

Half of the filtrate is checked for pH, which must be within the limits 6.8 - 7.1. The other half is tested for albumen, by boiling and adding 3% acetic acid. Protein, (albumen) should be present in large amounts (a +++ result). If both pH and protein tests are satisfactory, the flask of mixture is steamed for 35 minutes to coagulate the meat, and left overnight to cool and settle. The supernatant is then decanted with aseptic precautions, filtered aseptically through sterile filter paper and funnel into a sterile flask and then dispensed (also aseptically) into sterile 500 ml. screw-capped bottles. These are steamed for 15 minutes in case of accidental contamination, and stored at room temperature. For current use, the digest is dispensed with sterile precautions into 1 oz. vials and again steamed for

10 minutes.

6. <u>50% digest broth</u>:

To 1 volume of digest (v. above) add 1 volume of sterile 0.8% sodium glycerophosphate in distilled water, and adjust with sterile 2N Na_2CO_3 to pH 7.4. Steam 15 minutes.

7. <u>Digest agar</u>:

Medium no. 2 with 5% digest added while melted, just before pouring plates.

8. <u>20% blood agar</u>:

Human or rabbit blood (citrated), 60 ml., substituted for the human blood in 4, or added to sloppy agar as in 21.

9. <u>1% starch blood agar</u>:

10 g. soluble starch boiled in 100 ml. distilled water till the mixture clears. Whilst still warm, 30 ml. is added to medium 8 before pouring.

10. <u>1% maltose blood agar</u>:

20% maltose in distilled water is tyndallised (20 minutes steaming on each of 3 successive days) and stored in screw-capped bottles until ready for use. 15 ml. is added to medium number 4 before pouring. Other sugars were added in a similar manner.

11. <u>Agar-lined maltose blood agar</u>:

This is number 10 with 3 - 4 ml. hot melted ordinary agar poured on after setting of 10, and

allowed to spread evenly over the plate.

12. Cellophane-lined blood agar and maltose blood agar:

To 4 or 10 a 4-inch disk of wrapping cellophane or alternatively PT 300 cellophane (British Cellophane, Ltd.) which had been steam-sterilised, was added and pressed gently down on the medium.

13. Agar- and cellophane-lined medium:

12 plus 4 ml. agar lining as in 11 on top of the cellophane.

14. 0.25% blood plasma digest agar:

To melted digest agar (no. 7) at 50°C., 5% sterile human plasma and 0.25% human blood were added and plates poured.

15. 0.25% blood plasma digest agar with glucose:

1.25% of 20% glucose solution, and 2% of sterile 0.1% bromthymol blue were added to 14.

16. Horse, ox, sheep, or rabbit blood agar:

The same as 4 except that sterile citrated blood of the appropriate species is used instead of human blood.

17. Blood broth:

To ordinary broth (no. 1) add 6% or 0.5% human blood and tube out aseptically.

18. Maltose blood broth:

To no. 17 add maltose, 1.25% of 20% sterile solution.

19. Bijou blood agar:

3 ml. volumes of no. 4 are poured into sterile

$\frac{1}{4}$ oz. screw-capped bottles and allowed to solidify in the sloped position.

20. Blood agar broth:

Number 4 medium is pipetted with aseptic precautions while still molten, into 6" x 5/8" sterile tubes, care being taken to keep the tube and pipette vertical, so as to avoid spreading the blood agar up the sides of the tube. After this has set, 3 ml. PDB (no. 35) is pipetted aseptically on to the blood agar. The tubes are incubated for 48 hours at 37°C. before use.

21. Sloppy agar on blood agar:

This is no. 2 medium with the substitution of 0.05% New Zealand agar for the higher concentration normally used, and superimposed with sterile precautions while warm, on blood agar as in no. 20.

22. Glucose phosphate peptone water blood agar:

Glucose phosphate peptone water is made as in Mackie and McCartney, (1953, page 181), and superimposed with sterile precautions on blood agar as in no. 20.

23. Christensen's urea broth on blood agar:

Christensen's urea medium (Christensen, 1946) was made without the agar and superimposed on blood agar as in no. 20.

24. Nutrient gelatin on blood agar:

Nutrient gelatin was made as in Mackie and

McCartney (1953, page 153) except that ordinary broth (1) was used. The warm gelatin medium was tubed on to blood agar as in no. 20.

Media containing altered blood:

25. Levinthal's boiled blood agar: (Levinthal, 1918)

Medium no. 4, before being poured, is brought to the boil for a moment, then allowed to cool to about 60°C. before pouring plates.

26. Autoclaved blood agar:

Medium no. 4 while still liquid, is autoclaved in 150 ml. amounts at 120°C. for 30 minutes. Plates are poured while the medium is still hot.

27. Chocolate bijou agar slopes:

As no. 19 except that the bijou bottle containing the blood agar is steamed for ½ hour before sloping.

28. Boiled blood broth:

As no. 25 without agar.

29. Boiled blood agar with maltose and phenolphthalein phosphate for phosphatase test.

To medium no. 25, while still liquid, add 0.25% maltose (1.25% of the stock sterile 20% solution), together with 0.01% phenolphthalein diphosphate.

30. Boiled and autoclaved red cell extract digest agar:

Two samples of human blood are centrifuged and the red cells washed and packed. Peptone broth is

added, to the original blood volume. One sample is boiled till the red cells separate, centrifuged and the supernatant removed and steamed 15 minutes. 10% of this is then added to medium no. 7. The other sample is autoclaved for 30 minutes at 120°C., then treated the same way as the first.

31. Lysed blood agar, as for sulphonamide sensitivity tests:

See under no. 3.

Media containing chief blood constituents:

A. Plasma:

32. 5% digest broth:

To medium no. 1, 5% digest is added and the medium steamed 15 minutes.

33. 5% plasma digest agar (PDA):

To no. 7, 5% sterile human plasma is added before pouring plates.

34. Glucose PDA with indicator:

To no. 33 are added 0.25% sterile glucose and 0.005% sterile bromthymol blue (5% of 0.1% sterile solution), before pouring plates.

35. 5% plasma digest broth (PDB):

To no. 32, 5% sterile human plasma is added and the medium tubed out with sterile precautions.

36. 5% "glucose-free" plasma digest broth:

To no. 32, 5% human plasma, obtained from a special bleeding in which disodium citrate solution

alone was used as anti-coagulant, is added.

37. <u>Sugar fermentation media, based on no. 36.:</u>

The following sugars were used:

Glucose, lactose, saccharose, maltose, laevulose, galactose, dextrin and glycerol - all made up in 20% concentration in distilled water and tyndallised;

Dulcite was made up in 10% solution, starch was made up 10% fresh each time and glycogen was made in 5% solution and tyndallised.

The above sugars were added, to a final concentration of 1% or 0.25%, to medium no. 36, before aseptic tubing is carried out. Where method 2 of Chapter V is used for the inoculation of sugar media, plasma is omitted from the medium until the time of inoculation, and the opportunity is then taken of giving the tubes a final steaming for 10 minutes, to reduce the risk of contamination.

In the case of arabinose and xylose, 20% solutions are Seitz-filtered instead of tyndallised, as these sugars are easily decomposed by heating, and no final steaming was given after tubing.

38. <u>Seitz-filtered plasma digest broth (FPDB):</u>

As number 35 except that the plasma is Seitz-filtered before addition.

39. <u>Plasma digest glucose phosphate peptone water (PDGPPW):</u>

To glucose phosphate peptone water (Mackie and

McCartney, 1953) 5% plasma and 5% digest are added.

40. <u>5% plasma thioglycollate medium</u>:

See number 64.

B. <u>Serum</u>:

41. <u>Horse serum agar:</u>

This is medium no. 2 with 5% sterile horse serum added before pouring plates.

42. <u>Steamed horse serum agar</u>:

This is medium no. 41 steamed for 30 minutes before pouring plates.

43. <u>4% human serum digest agar</u>:

Human serum is made by adding 0.4% $CaCl_2$ to citrated plasma, and separating the serum from the resultant clot, with aseptic precautions. 4% is then added to medium no. 7 before pouring plates.

44. <u>Filtered 5% human serum agar</u>:

Serum is prepared as in no. 43, is then Seitz-filtered, and 5% is added to medium no. 7 before pouring plates.

45. <u>Amies and Jones' medium (serum yeast agar)</u>:

As in Amies and Jones (1957), but modified by substitution of bacto-casitone for the tryptose, 0.8% New Zealand agar for the 1.5% Japanese agar, and Lab Lemco 1% in distilled water instead of the infusion of beef heart. The phenol red was omitted except where sugars were included for fermentation testing. Human serum was used, either unfiltered

or Seitz-filtered.

46. <u>10% filtered serum digest agar (10%FSDA)</u>:

To medium no. 7, 10% Seitz filtered human serum is added before pouring plates. When sugar is incorporated, this is done as described under no. 37, before pouring plates, no final steaming being given.

47. <u>5% red cell digest agar</u>:

Sterile washed red cells from human blood are made up to the original blood volume with sterile saline and 5% added to medium no. 7 before pouring plates.

48. <u>Haemoglobin digest agar:</u>

i. Haemoglobin was prepared by the method given in Starling's Principles of Human Physiology (Lovatt Evans, 1947, page 486) involving the lysing of washed red cells and removal of the stromata. This was done with sterile precautions and 3%, 0.03% or 0.0003% of the resulting solution of haemoglobin was added to medium no. 7 before pouring plates.

ii. Pure haemoglobin was obtained from L.Light and Co. Ltd. One part Hb was ground into 39 parts of distilled water in a mortar until dissolved. It was then Seitz-filtered (with great difficulty) and 30 ml. filtrate added to 100 ml. medium no. 7 as before. This gave approximately 0.5% Hb in the

medium (the Hb did not completely dissolve and some was therefore held back by the filter).

49. Haem digest agar:

To prepare haem digest agar, crystalline haemin is first dissolved in distilled water in 0.8% concentration and Seitz-filtered. To 6 ml. of the sterile solution 0.25 ml. of 2% aqueous Na_2SO_3 solution (steam sterilised) is added. This causes the reduction of haemin to haem. The solution is then added to 150 ml. of digest agar (no. 7) before pouring plates, giving a final concentration of haem of 1/3000. This is roughly the same as in blood agar.

50. Haemin digest agar:

Pure haemin is dissolved in distilled water to 0.8% concentration and sterilised by Seitz-filtration. Six ml. of this is then added to digest agar (no. 7) before pouring plates. This gives a final concentration of 1/3000.

51. Haematin media: (X factor):

One part by weight of haematin (obtained from L. Light and Co. Ltd.) is dissolved in 100 parts by volume of N/10 NaOH, e.g. 10 mg. in 1.0 ml. This gives a dense blackish brown clear solution on heating over the bunsen. The solution is then diluted 1/10 to give a 1/1000 solution in N/100 NaOH, and sterilised by autoclaving.

The appropriate volumes of this sterile 0.1% solution are then added to the various media to give one of the following concentrations according to the experiment:- 1/600, 1/10,000, 1/20,000, 1/33,000, 1/200,000 and 1/2,000,000. The media can be sterilised by heat after the addition of X factor.

52. Media with added V factor:

V factor was supplied either as yeast extract or as pure co-enzyme I.

i. Yeast extract:

This was made as in Edward (1947) for all the media in which yeast extract was used during the investigation. 50 g. brewers' yeast was boiled in 200 ml. distilled water until the frothing ceased. The mixture was then centrifuged and the supernatant fluid Seitz-filtered.

ii. Co-enzyme I:

Obtained 95% pure from L. Light and Co. Ltd. One part by weight was dissolved in 1000 parts by volume of distilled water, e.g. 10 mg. in 10 ml. The solution was then sterilised by Seitz-filtering using a Hemmings filter. The appropriate volume of the 1/1000 solution was added to media, usually melted digest agar, with or without serum, to give either 1/50,000 or 1/66,000.

53. Red cell stroma digest agar:

Red cell stromata were prepared by the method

given in Starling's textbook (Lovatt Evans, 1947) page 486, with aseptic precautions throughout. After washing at least 3 times in sterile distilled water, the stromata were packed by centrifuging at 2000 r.p.m. for 10 minutes and 2% packed stromata added to medium number 7 before pouring plates.

Media without blood:

54. Solidified Cohen and Wheeler's medium (modified as suggested by Mazloum and Rowley, 1955).

Difco Bacto-casitone	10 g.
NaCl (Analar)	2.5 g.
KH_2PO_4 (Analar)	0.5 g.
$MgSO_4.7H_2O$ (Analar)	0.48 g.
Starch, soluble, powdered	1.5 g.
$CaCl_2$ (Analar, 1% solution	1.0 ml.
$CuSO_4.5H_2O$ (Analar, 0.05% solution)	1.0 ml.
Yeast extract, aqueous, fresh	50 ml.
Glutamic acid	5.0 g.
Charcoal (if used)	5.0 g.
New Zealand agar	9.0 g.
Distilled water	to 1000 ml.

Dissolve the first 4 ingredients in part of the water. Add the remaining substances and make up to 1 litre. Steam to melt agar and dissolve starch. Adjust pH to 7.2 - 7.3. Dispense in 100 ml. amounts in 150 ml. bottles. Autoclave at 10 p.s.i. for 15 minutes.

Prepare cystein hydrochloride 1% solution in distilled water.

$FeSO_4.7H_2O$ 1.25% in N/1000 HCl

Seitz-filter these to sterilise them. To each 100 ml. quantity of medium add 0.25 ml. sterile 1% cysteine HCl solution and 0.1 ml. $FeSO_4.7H_2O$ solution, with aseptic precautions. Pour plates as required.

55. Cohen and Wheeler (modified) plus catalase:

To no. 54 add 1/3000 crude beef catalase (Light's). A 1/20 solution was made of the catalase, in Na_2HPO_4 buffer as described by Mazloum and Rowley (1955). After centrifuging, the solution was membrane filtered. 0.67 ml. was then added to 100 ml. melted CW agar, at approximately 50°C. and plates poured.

56. 5% filtered serum digest agar plus catalase:

To 150 ml. medium no. 44, before pouring plates, 1 ml. sterile catalase solution was added and plates poured. The catalase was this time obtained from Oxo in ampoules, having been dissolved in phosphate buffer and sterilised by membrane filtration. It was freeze-dried shortly after receipt and later reconstituted in sterile distilled water for use. One ml. of the original solution decomposed approximately 35 g. H_2O_2 in 10 minutes at 25°C.

Media for isolation of PPLO:

57. Soft blood agar (0.5% agar):

To 100 ml. medium no. 2 (ordinary agar) before pouring plates, add 80 ml. sterile broth, 10 ml. human blood and 9 ml. digest, mix and pour plates thickly. Care must be taken not to empty the medium out of the plate as it is as soft as practicable for plate use.

58. Soft blood agar with thallium acetate and penicillin:

To no. 57 is added 1/8000 thallium acetate, using a previously autoclaved 10% solution in distilled water, and plates are then poured. Penicillin solution, 1000 units/ml. is made up in sterile water, 2 drops spread across each half of the plate and allowed to dry.

59. 20% plasma thallium acetate plates (v. Edward, 1947):

To ordinary agar (no. 2) 1/8000 thallium acetate is added while melted, and stock bottles are dispensed for later use. To prepare plates, melt down the thallium acetate agar, add 5% digest, 10% yeast extract and 20% human plasma, or horse serum. Plates are poured, incorporating a sterile glass slide, so that the surface of the slide is covered to a depth of 1-2 mm. Penicillin is sometimes added as in no. 58, before use.

60. Thallium acetate sloppy agar in tubes:

Ordinary agar, (no. 2) is made, except that

0.15% New Zealand agar is used instead of 0.8 - 0.9%. To the melted and cooled medium, 20% human plasma (or horse serum) is added, together with 10% yeast extract, 1/2000 thallium acetate and 60 units/ml. of penicillin. The medium is then dispensed with sterile precautions into 5" x ½" sterile tubes with cotton wool stoppers.

61. <u>Thioglycollate medium with 0.15% New Zealand agar</u>:

To digest broth (no. 32) add 0.1% thioglycollic acid, 0.15% New Zealand agar and 0.1% glucose. Soak overnight and bring to pH 7.3 after steaming. Tube in 8 ml. amounts in 5" x ½" tubes and steam sterilise.

62. <u>Thioglycollate medium with 0.05% N.Z. agar</u>:

As no. 61 except that the agar concentration is 0.05%.

63. <u>Oxoid thioglycollate (fluid) medium</u>:

Formula given by manufacturers:

Bacteriological yeast extract (Oxoid)	5 g.
Tryptone (Oxoid)	15 g.
Dextrose	5.5 g.
Sodium thioglycollate	0.5 g.
Sodium chloride	2.5 g.
l-cystine hydrochloride	0.5 g.
Agar-agar (Oxoid)	0.5 g.
Resazurin	0.001 g.

pH 7.1 approximately.

One tablet is soaked in 10 ml. cold distilled water for 15 minutes and the tube autoclaved at 10 p.s.i. for 20 minutes.

64. Oxoid thioglycollate (fluid) medium plus 5% plasma:

Same as no. 63, with the addition of 5% sterile human plasma.

65. Cholesterol digest agar:

Into medium no. 7, 1/100,000 cholesterol is incorporated, by adding 0.1 ml. of a 1% alcoholic solution of cholesterol to 100 ml. melted medium and mixing thoroughly. The medium is then steamed for 45 minutes and plates poured.

66. Lecithin digest agar:

Lecithin is dissolved by shaking 10 mg. in 1 ml. distilled water with grade 3 ballotini on the Kahn shaker for 1 hour. The solution is then diluted to 1/1000. One per cent. of this solution is added to medium no. 7 when melted, and the medium steamed as above and plates poured.

67. Cephalin digest agar:

One mg. cephalin is dissolved in 1 ml. distilled water as for lecithin and the resulting 1/1000 solution added in the same concentration to the same medium as in nos. 65 and 66.

68. Nucleic acid digest agar:

1.36 g. nucleic acid is dissolved in 50 ml. N/9 NaOH, by stirring at room temperature. The

solution is then made up to 136 ml. with distilled water and Seitz-filtered. 2% of this solution is added to medium no. 7 before pouring plates, giving a final concentration of nucleic acid of 1/5000.

69. Cholesterol lecithin cephalin nucleic acid digest agar:

This consists of a combination of the above 4 media, the first three substances being added before the final steaming, the nucleic acid solution after, just before pouring plates.

70. Formula of Pruvagol:

Na salt of diamino-disulphomethyl fuchsonium sulphonate.......................... 0.06%

Na biborate.......................... 0.4%

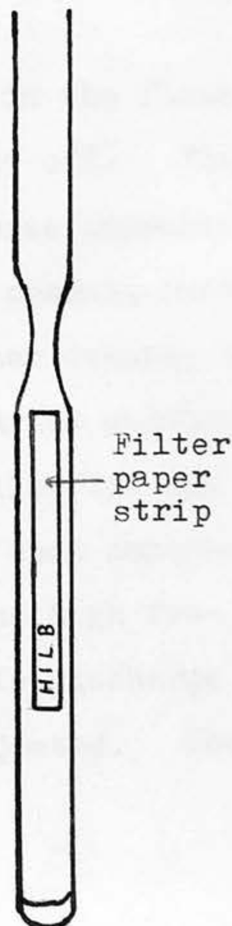
Recommended by Hart and Brown (1952) for treatment of vaginal moniliasis, and by the makers (Camden) for "pleomorphic cocco-bacilli" present in cervicitis of non-specific type.

Technique of freeze-drying:

At first a 48-hour aerobic 37°C. culture in blood agar broth was used. Later it was found that better results were obtained by emulsifying a 48-hour blood agar culture in 10% plasma broth.

For the first half of the investigation, the laboratory did not possess a freeze-drier, and the following technique was devised to overcome this lack.

Type L freeze-drying ampoules, 7 - 7.5 x 99 - 101 mm., as supplied through Edwards High Vacuum Company, were used, and into each ampoule a strip of Whatman's number 1 filter paper, 1" x 1/8", bearing the typed designation of the strain, was introduced. The cotton-stoppered ampoules were autoclaved and dried in vacuo in a desiccator over P_2O_5. They were then drawn out in a bunsen flame so as to give a neck of about 1/8" in diameter. Using a Pasteur pipette, charged with 4 or 5 drops of HILB culture, the filter paper was brought towards the neck of the ampoule and made to adhere to the wall by adding a drop of culture to it. Three to four drops of culture were then placed in the bottom of the ampoule (v. diagram).



The ampoules were then arranged as vertically as possible in the desiccator (medium vacuum type) over P_2O_5, and the lid replaced. All joints were kept clean and lightly greased with high vacuum grease (Apiezon). The desiccator was evacuated with an Edwards high vacuum pump, connected via a stopcock to the desiccator. When the material boiled, and frothed up, the froth was caught on the filter

paper and did not rise through the neck of the ampoule. Freezing then occurred, and pumping was continued until a vacuum of 0.01 mm. Hg was reached, as registered by the Vacustat gauge. The stopcock was then turned off and the pump disconnected. Next day, the ampoules were sealed individually in vacuo. The neck of the ampoule is too wide by this technique to seal off in one movement, and so the flame was allowed to impinge only long enough to produce a dimple. The ampoule was then removed from the flame, rotated and a second and a third dimple made round the circumference of the neck, as in diagram.

Cross-section of ampoule neck:

The whole neck was now immersed in the flame and the ampoule drawn out until sealed off. This gave a strong, undistorted seal. Three ampoules were used for each strain, one being opened, reconstituted and cultured immediately after drying, in order to check the viability of the dried culture. After a day or two on the bench to allow for any leakage due to a defect in the seal, each ampoule (2 of each strain) was tested with the high frequency tester, and if a blue or purple discharge was not obtained, the ampoule was rejected. Sound

ampoules were stored in the refrigerator, the sealed end being protected by a piece of rubber tubing.

During the second half of the investigation, a centrifugal freeze-drier was used, and there was not the same necessity to "stick" the filter paper to the upper end of the ampoule body, but otherwise the technique was the same.

For recovery of the culture, the ampoule was cracked open, after filing, with a hot glass rod. The inoculum was reconstituted in broth and plated out on blood agar plates in duplicate. After 3 days aerobic incubation at 37°C., single colonies were picked, 3 or 4 from each of the two plates, on to blood agar and the cultures incubated for 48 hours at 37°C. A full scale growth was not generally achieved until the 3rd. or 4th. successive culture after opening the ampoule. For each ampoule opened, 2 were put up again, as soon as good enough growth occurred, one being tested for viability after freeze-drying, the remaining one being used to keep the supply of ampoules to the minimum of 2.

Statistical calculations:

The χ^2 table for the incidence of HILB in all clinical groups is seen below (Table 79).

TABLE 79.

X^2 for HILB incidence in the different clinical groups, taken separately.

Groups	PP	PC	AN	G	GC	PM	PMC	Total
Observed no. HILB +ve.	32	8	12	18	9	7	4	90
Expected no. ⊛	17.5	10.1	17.3	16.3	16.3	7.0	5.4	90
Observed minus expected no.	14.5	-2.1	-5.3	1.7	-7.3	0	-1.4	
Difference^2	210	4.4	28	2.9	53	0	2	
$\text{Difference}^2 \div$ expected no.	12.0	0.44	1.62	0.18	3.25	0	0.37	
Observed no. HILB -ve.	13	18	32	24	33	11	10	141
Expected no.	27.5	15.9	26.7	25.7	25.7	11.0	8.6	141
Observed minus expected no.	-14.5	2.1	5.3	-1.7	7.3	0	1.4	
Difference^2	210	4.4	28	2.9	53	0	2	
$\text{Difference}^2 \div$ expected no.	7.64	0.28	1.05	0.11	2.06	0	0.23	

⊛ Overall proportion of HILB positive cases is 38.9% (v. Table 2).

Expected number, on basis that no difference exists between the clinical groups, is 38.9% of the number in each group, e.g. of 45 in the case of the PP group.

$X^2 =$ Sum of all values of $\text{Difference}^2 \div$ Expected Number $= 29.23$.

Number of independent groups is 6, $\therefore$ from Fisher's X^2 table, $p = \leqslant 0.01$

The X^2 calculation for the control groups, including ante-natal, is given in Table 80.

TABLE 80.

X^2 for HILB incidence among the control groups:

Groups	AN	PC	GC	PMC	Total
Observed no. HILB +ve.	12	8	9	4	33
Expected no.* HILB +ve.	11.5	6.8	11.0	3.7	33
Observed minus expected no.	0.5	1.2	-2.0	0.3	
Difference^2	0.25	1.44	4.0	0.09	
Difference^2 ÷ Expected no.	0.02	0.21	0.36	0.02	
Observed no. HILB -ve.	32	18	33	10	93
Expected no.* HILB -ve.	32.5	19.2	31	10.3	93
Observed minus expected no.	-0.5	-1.2	2.0	-0.3	
Difference^2	0.25	1.44	4.0	0.09	
Difference^2 ÷ Expected no.	0.01	0.08	0.13	0.01	

*

From table 2, the total number of cases in the 4 groups concerned is 126, of which 33 are HILB positive (i.e. 26.2%). For HILB negative cases (126 - 33 = 93), the corresponding proportion is 73.8%. Expected numbers are therefore 26.2% (or 73.8%) of the number of cases in each group (v. Table 2).

$X^2 = 0.84$, $n = 3$ ∴ from Fisher's table

$p = >0.8$

Example of calculation of Standard Error of Difference between two proportions:

Difference between proportion of HILB positive cases in PP and PC groups.

Standard Error of the Difference

$$= \sqrt{\frac{p \times q}{n_1} + \frac{p \times q}{n_2}}$$

where p = proportion of HILB positive in combined PP + PC, $\frac{40}{71} \times 100 = 56.5\%$.

where q = proportion of HILB negative in combined PP + PC, (100 - 56.5)% = 43.5%

n_1 = number of PP cases = 45

n_2 = number of PC cases = 26

$$\therefore \text{S.E.} = \sqrt{\frac{56.5 \times 43.5}{45} + \frac{56.5 \times 43.5}{26}}$$

$$= \pm 12.2\%$$

Observed difference = $(71.0 - 30.8)\%$

$= 40.2\%$

$= 3\frac{1}{4} \times S.E.$

The Standard Errors of the differences between proportion of HILB positive cases in G and GC; G and (PC + GC + PMC); yeast infections and total cases without yeast infection; PPLO isolations from HILB positive cases and from HILB negative cases; PPLO isolations from HILB positive and HILB negative cases of pH >5.4; were all calculated in the same way as above.

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