A Taxonomic Study of the Genus *Haemophilus*, with the Proposal of a New Species

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(Received 20 February 1975; revised 29 September 1975)

SUMMARY

A collection of 426 *Haemophilus* strains isolated from people with infectious diseases and from the normal flora of mucous membranes in humans and various animal species was studied in an attempt to revise and improve the taxonomy of the genus *Haemophilus*. The examinations included the determination of a number of biochemical and physiological properties, of which several had not previously been applied to the taxonomy of haemophili. The resulting data revealed many hitherto unrecognized characters of taxonomic significance, and several of the species can now be more accurately defined. The classification presented is supported by the DNA base composition of a large number of representative strains. A diagnostic key to the different taxa is presented. *Haemophilus influenzae* and *H. parainfluenzae* have been subdivided into a number of biotypes. It is possible to demonstrate a relationship between the individual biotypes of *H. influenzae* and the origin of the strains assigned to them. The results indicate that *H. aegyptius*, *H. parahaemolyticus* and *H. paraphrohaemolyticus* do not merit specific status. Four unnamed taxa of V-factor-dependent haemophili have been recognized. The name *Haemophilus segnis* is proposed for one of these taxa, which consists mainly of strains isolated from the human oral cavity. It is demonstrated that the name *H. ducreyi* has been used for different groups of bacteria, and that only one of these groups can legitimately be assigned to the genus *Haemophilus*. Haemolytic V-factor-dependent strains from swine, previously included in *H. parahaemolyticus*, are significantly different from strains of human origin and should be named *H. pleuropneumoniae*. None of the strains from swine and fowls were haemin-dependent. The relationships of these strains to the species *H. suis* and *H. gallinarum*, and to *H. parasuis* and *H. paragallinarum* are discussed. *Haemophilus piscium* is shown not to belong to the genus *Haemophilus*. The taxonomic position of *H. aphrophilus* is uncertain and its possible relationship to *Actinobacillus actinomycetemcomitans* requires further study. The positive correlation found between the ecology of the strains studied and their affiliation with the different taxa is discussed.

INTRODUCTION

The genus *Haemophilus* is based on the 'influenza bacillus' discovered and described by Pfeiffer in 1892. In spite of the loss of interest in this group of bacteria immediately after 1933, following the discovery of the influenza virus, interest has increased recently, and haemophili are now recognized as pathogens in their own right and implicated in a wide range of pathological conditions in man and various animal species.

The circumscription of the genus *Haemophilus* has been gradually but significantly contracted since the original proposals of the American Committee on Classification and Nomenclature (Winslow *et al.*, 1920). At present there is general agreement that the genus

should be restricted to Gram-negative rods or coccobacilli with a requirement for haemin or certain other porphyrins (X-factor) and/or NAD (V-factor) or other definable coenzyme-like substances (Zinnemann, 1967). Parallel with the narrowing of the genus, however, the number of proposals for new species of *Haemophilus* has been increasing. Whereas the 7th edition of *Bergey's Manual of Determinative Bacteriology* (1957) referred to 15 *Haemophilus* species, the number has been decreased by 4 in the recent 8th edition (1974). Concomitantly, however, 5 of the older species are now designated *species incertae sedis*. Of the 19 species referred to, 5 require both X- and V-factors (*H. influenzae*, *H. suis*, *H. haemolyticus*, *H. gallinarum*, *H. aegyptius*), 6 require V-factor only (*H. parainfluenzae*, *H. parasuis*, *H. parahaemolyticus*, *H. paraphrophilus*, *H. paragallinarum*, *H. paraphrohaemolyticus*), 5 require X-factor only (*H. haemoglobinophilus*, *H. influenzaemurium*, *H. aphrophilus*, *H. ducreyi*, *H. ovis*), 2 require factors as yet unidentified (*H. putriorum*, *H. citreus*), and the last (*H. piscium*) requires diphosphothiamine or ATP but neither haemin nor NAD for growth. Little is known about several of these species and no representative strains are available for study.

In the intervening period between the last two editions of *Bergey's Manual of Determinative Bacteriology*, some additional species have been described. These include *Haemophilus pleuropneumoniae* Shope 1964, an organism which is the causative agent of a characteristic, frequently fatal, contagious pleuropneumonia in swine. Requiring V-factor only and being haemolytic, this organism is considered to be a synonym of *Haemophilus parahaemolyticus* Pittman 1953 (Nicolet, 1968; Zinnemann, 1971; *Bergey's Manual of Determinative Bacteriology*, 1974). An organism associated with encephalitis of cattle has been referred to as *Haemophilus somnus* (Brown, Dierks & Dillman, 1970) as proposed by Bailie (1969) or *Haemophilus somnifer* (Miles, Anthony & Dennis, 1972), but obviously does not meet the criteria for inclusion in the genus *Haemophilus* (Bailie, Coles & Weide, 1973). Likewise, *Haemophilus agni* (Kennedy *et al.*, 1958), an organism implicated in septicæmia of lambs, is no longer considered to be a member of *Haemophilus* (Zinnemann, 1971). Although still under discussion (Criswell *et al.*, 1971), *Haemophilus vaginalis* Gardner & Dukes 1955 is now usually considered Gram-positive and consequently as belonging to some other genus (Zinnemann & Turner, 1963; Reyn, Birch-Andersen & Lapage, 1966; Jones & Weitzman, 1971; *Bergey's Manual of Determinative Bacteriology*, 1974).

The study of biochemical reactions of haemophili has been retarded by the difficulty experienced in preparing media which allow copious growth. Few biochemical tests, generally applicable to other bacterial groups, have been used with success in studies of haemophili. The consequent paucity of comparable data, and reliance on single or few characters of uncertain taxonomic significance when establishing new species, has not only hampered the development of a satisfactory classification but has also rendered an exact identification of *Haemophilus* strains difficult.

Finally, inadequate recognition of the fact that haemophili are a constituent of the normal flora of the mucous membranes of the human throat (Dawson & Zinnemann, 1952; Kilian, Heine-Jensen & Bülow, 1972), oral cavity (Fleming & Maclean, 1930; Sims, 1970) and sometimes genital tract (Lapage, 1961; *Bergey's Manual of Determinative Bacteriology*, 1974) has resulted in a lack of incentive for a closer study of virulence factors of pathogenic haemophili, and impeded the evaluation of the significance of results obtained in clinical bacteriological laboratories. The same problems also apply to the veterinary field.

I have attempted to revise and improve the taxonomy of the genus *Haemophilus*. A large collection of strains, heterogenous in origin and pathogenicity, has been studied using a number of methods not previously applied to this group of bacteria. The validity of the

resulting classification has been supported by the determination of the DNA base composition of a large number of strains.

METHODS

Selection of bacterial strains

The general prerequisite for including a strain in the survey as a member of the genus *Haemophilus* has been a negative Gram reaction, a coccobacillus- or rod-shape, and a proven requirement for either or both of the two growth factors NAD (V-factor) and haemin (X-factor) or for precursors to these. The only exception to the latter has been strains resembling *Haemophilus aphrophilus*. Strains maintained in culture collections have been included as far as possible and, with named strains mentioned in the literature, have been included without restrictions. The strains were collected from many sources in an attempt to obtain as diverse a collection as possible. In addition to strains isolated from various infectious diseases, both human and veterinary, a large proportion were isolated from the normal flora of mucous membranes. These, as well as strains from conjunctivitis, were selected from a larger number of strains after a preliminary examination. The relative composition of the selected strain materials does not reflect the composition of the original materials, which are described in detail elsewhere (Kilian & Schiøtt, 1975; Kilian *et al.*, 1976).

The collection includes 426 strains received and/or identified as *Haemophilus*; for comparison, 12 strains of the species *Actinobacillus actinomycescomitans*, *Eikenella corrodens* and *Pasteurella multocida* were also studied. Apart from recent isolates from clinical bacteriological laboratories in Denmark, all strains were received as freeze-dried cultures. On receipt, the strains were checked for purity, and after the lowest possible number of subcultures, Levinthal broth cultures were frozen in sealed glass ampoules at -70°C . An ampoule was opened whenever a new series of tests was performed.

A code number (HK) has been assigned to each strain of the collection. Table 1 lists the strains under the species to which they have been assigned as a result of this study. Table 2 summarizes the sites of origin of the strains.

Culture methods

Chocolate agar [10%, v/v, heated, defibrinated horse blood in blood agar base (Difco)] was used for maintaining strains, for inoculum cultures for all test media and for growth of strains for DNA extraction. Levinthal's agar (Alexander, 1958) was used for the detection of capsule production. Unless otherwise stated, agar plates were incubated in anaerobic jars evacuated then filled with air plus 10% carbon dioxide. Levinthal 'stock' (Turk & May, 1967) was used whenever fluid cultures were needed, the basal broth being brain-heart infusion (Difco). The medium will be referred to as Levinthal broth.

Stock cultures and inoculated test media were incubated at 37°C , except for strains of *H. piscium* which were incubated at 22°C .

Morphology and staining

Colonial morphology was described from 48 h cultures on chocolate agar. A saline suspension of a colony was examined by phase-contrast microscopy for shape and motility, and a heat-fixed film was Gram-stained by Hucker's modification (Hucker & Conn, 1923).

Table 1. *Strains studied, listed according to the classification resulting from this study*

Study reference number	Received as	Isolated from	Source* and strain designation
<i>Haemophilus influenzae</i> , biotype I			
HK2, 6, 20, 27, 41, 43, 61, 66, 76	<i>Haemophilus</i> sp.	Pharynx	Kilian <i>et al.</i> (1972)
HK141, 144	<i>Haemophilus</i> sp.	Human saliva	Kilian & Schjøtt (1975)
HK162	<i>H. influenzae</i>	Epiglottitis	W. Frederiksen (100672)
HK163	<i>H. influenzae</i>	Epiglottitis	K. Zinnemann (Rustforth)
HK171-179	<i>H. influenzae</i>	Meningitis	H. Lautrop (strains AB1872 and AB1876 to AB1883, respectively)
HK180-191	<i>H. influenzae</i>	Meningitis	Engbaek (1949) (strains 383, 392, 844, 868, 875, 1035, 1065, 1266a, 1379, 4857, 984 and sp1000, respectively)†
HK192	<i>H. influenzae</i>	Meningitis	W. Frederiksen (351483)
HK193-205	<i>H. influenzae</i>	Meningitis	P. Bülow
HK213-216	<i>H. influenzae</i>	Human blood	P. Bülow
HK217, 220	<i>H. influenzae</i>	Sinusitis	P. Bülow
HK224, 226	<i>H. influenzae</i>	Otitis media	V. F. Thomsen†
HK234, 235, 236, 243, 246, 247, 254	<i>H. influenzae</i>	Otitis media	P. Bülow
HK293	<i>Haemophilus</i> sp.	Conjunctivitis	C. R. Dawson (539) (Tunisia)
HK297	<i>H. influenzae</i>	Pneumonia	P. Bülow
HK390	<i>H. influenzae</i>	Respiratory infection	NCTC8465 (serotype a)
HK391	<i>H. influenzae</i>	Meningitis	NCTC8466 (serotype a)
HK392	<i>H. influenzae</i>	Meningitis	NCTC7279 (serotype b)
HK393	<i>H. influenzae</i>	Meningitis	NCTC8467 (serotype b)
HK395	<i>H. influenzae</i>	—	CIP5423 (Rb)
HK403	<i>H. influenzae</i>	Meningitis	NCTC8473 (serotype f)
<i>Haemophilus influenzae</i> , biotype II			
HK1, 4, 9, 11, 13, 21, 24, 25, 28, 30, 37, 38, 42, 46, 50, 54, 58, 62, 63, 68	<i>Haemophilus</i> sp.	Pharynx	Kilian <i>et al.</i> (1972)
HK123	<i>Haemophilus</i> sp.	Human saliva	Kilian & Schjøtt (1975)
HK166, 168	<i>Haemophilus</i> sp.	Conjunctivitis	N. Hegazy (Cairo)†
HK206, 208	<i>H. influenzae</i>	Meningitis	P. Bülow
HK210-212	<i>H. influenzae</i>	Meningitis	Engbaek (1949) (strains 865, 870 and 876, respectively)†
HK218, 219, 221, 222	<i>H. influenzae</i>	Sinusitis	P. Bülow
HK225, 227, 231, 232	<i>H. influenzae</i>	Otitis media	V. F. Thomsen†
HK237, 242, 244, 245, 248	<i>H. influenzae</i>	Otitis media	P. Bülow
HK263	<i>Haemophilus</i> sp.	Conjunctivitis	C. H. Mordhorst (AB1785)†
HK264, 266, 268	<i>Haemophilus</i> sp.	Conjunctivitis	C. H. Mordhorst
HK271	<i>Haemophilus</i> sp.	Conjunctivitis	C. H. Mordhorst (AB1905)†
HK273	<i>Haemophilus</i> sp.	Conjunctivitis	P. Bülow
HK274, 275, 283, 285	<i>Haemophilus</i> sp.	Conjunctivitis	C. H. Mordhorst (strains AB1862, AB1870, AB1857 and AB1946, respectively)†
HK287-290	<i>Haemophilus</i> sp.	Conjunctivitis	C. R. Dawson (strains 443, 518, 873 and 839 respectively) (Tunisia)
HK298, 299	<i>H. influenzae</i>	Pneumonia	P. Bülow
HK300, 301, 302	<i>Haemophilus</i> sp.	Chronic bronchitis	—
HK342	<i>H. influenzae</i>	—	Engbaek (1949) (Ruggier)†
HK389	<i>H. influenzae</i>	—	NCTC8143

Study reference number	Received as	Isolated from	Source* and strain designation
<i>Haemophilus influenzae</i> , biotype II (cont.)			
HK396	<i>H. influenzae</i>	Sputum	NCTC8469 (serotype c)
HK402	<i>H. influenzae</i>	Pus, mastoid	NCTC7918 (serotype f)
<i>Haemophilus influenzae</i> , biotype III			
HK7, 56, 57, 70, 71, 72, 73	<i>Haemophilus</i> sp.	Pharynx	Kilian <i>et al.</i> (1972)
HK207	<i>H. influenzae</i>	Meningitis	P. Bülow (non-capsulated)
HK241	<i>H. influenzae</i>	Otitis media	P. Bülow
HK265, 267, 269	<i>Haemophilus</i> sp.	Conjunctivitis	C. H. Mordhorst
HK270	<i>Haemophilus</i> sp.	Conjunctivitis	C. H. Mordhorst (AB1888)†
HK272	<i>Haemophilus</i> sp.	Conjunctivitis	P. Bülow
HK277-279, 284, 286	<i>Haemophilus</i> sp.	Conjunctivitis	C. H. Mordhorst (strains AB1866, AB1886, AB1884, AB1901 and AB1890, respectively)†
HK291, 292, 294, 295	<i>Haemophilus</i> sp.	Conjunctivitis	C. R. Dawson (strains 415, 864, 614 and 429, respectively)†
HK367	<i>H. aegyptius</i>	Conjunctiva	NCTC8502; suggested working type (Sneath & Skerman, 1966)
HK368	<i>H. aegyptius</i>	Conjunctiva	NCTC8134
HK369	<i>H. aegyptius</i>	Conjunctiva	NCTC8135
HK387	<i>H. influenzae</i>	—	NCTC4560; suggested working type (Sneath & Skerman, 1966)
HK388	<i>H. influenzae</i>	Respiratory tract	NCTC4842
<i>Haemophilus influenzae</i> , biotype IV			
HK17, 32, 44, 69	<i>Haemophilus</i> sp.	Pharynx	Kilian <i>et al.</i> (1972)
HK209	<i>H. influenzae</i>	Meningitis	Engbaek (1949) (204)†
HK343	<i>H. influenzae</i>	—	Engbaek (1949) (Dingle)†
HK394	<i>H. influenzae</i>	Meningitis	NCTC8468 (serotype b)
HK397	<i>H. influenzae</i>	Throat	NCTC8470 (serotype d)
HK398	<i>H. influenzae</i>	—	CIP5424 (Rd)
HK399	<i>H. influenzae</i>	—	NCTC10479 (serotype e ₁ e ₂)
HK400	<i>H. influenzae</i>	—	NCTC8455 (serotype e ₂)
HK401	<i>H. influenzae</i>	—	NCTC8472 (serotype e ₁ e ₂ , and degraded e ₂)
<i>Haemophilus influenzae</i> , biotype V			
HK223, 233	<i>H. influenzae</i>	Otitis media	V. F. Thomsen (AB1928 and AB1927, respectively)†
HK238, 239, 240, 249, 250, 251, 252, 253	<i>H. influenzae</i>	Otitis media	P. Bülow
<i>Haemophilus influenzae</i> , unclassified strains			
HK39	<i>Haemophilus</i> sp.	Pharynx	Kilian <i>et al.</i> (1972)
HK280, 281	<i>Haemophilus</i> sp.	Conjunctivitis	H. Lautrop (strains AB1863 and AB1865, respectively)
<i>Haemophilus haemolyticus</i>			
HK5, 12, 29, 33, 45, 53, 59, 75	<i>Haemophilus</i> sp.	Pharynx	Kilian <i>et al.</i> (1972)
HK386	<i>H. haemolyticus</i>	Sputum	NCTC10659
<i>Haemophilus haemoglobinophilus (canis)</i>			
HK365	<i>H. canis</i>	Prepuce, dog	W. Frederiksen (P301)←J. E. Smith (404)
HK375	<i>H. canis</i>	Prepuce, dog	NCTC1659; cotype (Sneath & Skerman, 1966)

Study reference number	Received as	Isolated from	Source* and strain designation
<i>Haemophilus haemoglobinophilus (canis) (cont.)</i>			
HK376	<i>H. canis</i>	—	NCTC8540
HK377	<i>H. canis</i>	Dog	NCTC10619
HK378	<i>H. canis</i>	Prepuce, dog	ATCC19416; same as NCTC1659, but see comment
Unclassified strain			
HK431	<i>Haemophilus</i> sp.	Perianal abscess	NCTC10555, Ryan (1968)
<i>Haemophilus ducreyi</i>			
HK344-350	<i>H. ducreyi</i>	Soft chancre	Reymann (1951) (strains II, IX, X XI, 129, 133 and 134 respectively)‡
HK379	<i>H. ducreyi</i>	—	CIP542
HK380	<i>H. ducreyi</i>	—	CIP A75
<i>Haemophilus parainfluenzae</i> , biotype I			
HK18, 47, 79	<i>Haemophilus</i> sp.	Pharynx	Kilian <i>et al.</i> (1972)
HK82, 86, 88, 89, 90, 91, 92, 93, 94, 96, 100, 105, 109, 110, 111, 115, 116, 118, 119, 124, 128, 130, 132, 133, 134, 136, 139, 140, 145, 146, 150, 151, 155, 156, 158, 160	<i>Haemophilus</i> sp.	Human saliva	Kilian & Schiøtt (1975)
HK256, 257, 259	<i>Haemophilus</i> sp.	Vagina	—
HK282	<i>H. parainfluenzae</i>	Conjunctivitis	H. Lautrop (fsk3567)
HK409	<i>H. parainfluenzae</i>	Septic finger	NCTC7857
HK416	<i>H. paraphrophilus</i>	Trachea	NCTC10558; cotype (strain 'Haywood')
<i>Haemophilus parainfluenzae</i> , biotype II			
HK3, 10, 14, 16, 23, 26, 31, 34, 36, 40, 49, 55, 60, 64, 67, 77, 78	<i>Haemophilus</i> sp.	Pharynx	Kilian <i>et al.</i> (1972)
HK81, 95, 97, 98, 99, 101, 102, 103, 104, 107, 108, 113, 117, 120, 122, 125, 138, 142, 152	<i>Haemophilus</i> sp.	Human saliva	Kilian & Schiøtt (1975)
HK255, 260, 262	<i>Haemophilus</i> sp.	Urethra	—
HK331	<i>H. parahaemolyticus</i>	Pus, oral cavity	W. Sims (21)
HK332	<i>H. parahaemolyticus</i>	Dental plaque	W. Sims (28)
HK335	<i>H. parahaemolyticus</i>	Human saliva	W. Sims (strains 31 and 32, respectively)
HK339	<i>H. haemolyticus</i>	—	J. Frazer←M. Pittman (734)
HK410	<i>H. parainfluenzae</i>	Human saliva	NCTC10665
<i>Haemophilus parainfluenzae</i> , biotype III			
HK8, 19, 22, 48, 51, 52, 65, 74	<i>Haemophilus</i> sp.	Pharynx	Kilian <i>et al.</i> (1972)
HK112, 121, 126, 137	<i>Haemophilus</i> sp.	Human saliva	Kilian & Schiøtt (1975)
HK169	<i>Haemophilus</i> sp.	Conjunctivitis	N. Hegazy (Cairo)
HK258	<i>Haemophilus</i> sp.	Vagina	—

Study reference number	Received as	Isolated from	Source* and strain designation
<i>Haemophilus parainfluenzae</i> , biotype III (cont.)			
HK261	<i>Haemophilus</i> sp.	Urethra	—
HK276	<i>H. parainfluenzae</i>	Conjunctivitis	H. Lautrop (AB1852)
HK303, 304, 305, 312	<i>Haemophilus</i> sp.	Dental plaque	Kilian & Schiøtt (1975)
HK330	<i>H. parahaemolyticus</i>	Tooth abscess	W. Sims (17)
HK333	<i>H. parahaemolyticus</i>	Pus, oral cavity	W. Sims (29)
HK334	<i>H. parahaemolyticus</i>	Human saliva	W. Sims (strains 30 and 33, respectively)
HK338	<i>H. parahaemolyticus</i>	Pus, oral cavity	W. Sims (34)
HK385	<i>H. haemolyticus</i>	Pulmonary infection	NCTC8479; suggested working type (Sneath & Skerman, 1966)
HK404	<i>H. parahaemolyticus</i>	Throat	NCTC10794
HK411	<i>H. paraphrohaemolyticus</i>	Sputum	NCTC10670; holotype
HK412	<i>H. paraphrohaemolyticus</i>	Sputum	NCTC10671; paratype
HK413	<i>H. paraphrohaemolyticus</i>	Throat	NCTC10672; paratype
HK444	<i>Haemophilus</i> sp.	Rat, oral cavity	—
<i>Haemophilus parainfluenzae</i> , unclassified strains			
HK15	<i>Haemophilus</i> sp.	Pharynx	Kilian <i>et al.</i> (1972)
HK80, 147	<i>Haemophilus</i> sp.	Human saliva	Kilian & Schiøtt (1975)
HK408	<i>H. parainfluenzae</i>	Human tongue	NCTC4101
<i>Haemophilus paraphrophilus</i>			
HK83, 85, 143, 159	<i>Haemophilus</i> sp.	Human saliva	Kilian & Schiøtt (1975)
HK296	<i>Haemophilus</i> sp.	Chronic bronchitis	—
HK306, 318, 319, 321	<i>Haemophilus</i> sp.	Dental plaque	Kilian & Schiøtt (1975)
HK414	<i>H. paraphrophilus</i>	Parietal abscess	NCTC10556; cotype
HK415	<i>H. paraphrophilus</i>	Paronychia	NCTC10557; holotype
<i>Haemophilus aphrophilus</i>			
HK308, 309, 310, 311, 315	<i>Haemophilus</i> sp.	Dental plaque	Kilian & Schiøtt (1975)
HK322	<i>H. aphrophilus</i>	Endocarditis	W. Frederiksen (P404)
HK323	<i>H. aphrophilus</i>	Gingiva	W. Frederiksen (P406)
HK324	<i>H. aphrophilus</i>	Abdominal wound	W. Frederiksen (P527)
HK325	<i>H. aphrophilus</i>	Joint infection	W. Frederiksen (P536)
HK326	<i>H. aphrophilus</i>	Sinusitis pus	W. Frederiksen (P537)
HK327	<i>H. aphrophilus</i>	Crural ulcer	W. Frederiksen (334542)
HK329	<i>H. aphrophilus</i>	Endocarditis	Dorff & Kilian, (1974)
HK371	<i>H. aphrophilus</i>	Endocarditis	NCTC5886; holotype
HK372–374	<i>H. aphrophilus</i>	Endocarditis	NCTC5906, NCTC5907 and NCTC5908, respectively)
<i>Haemophilus segnis</i> , sp.nov.			
HK35	<i>Haemophilus</i> sp.	Pharynx	Kilian <i>et al.</i> (1972)
HK84, 87, 106, 114, 127, 129, 131, 135, 149, 161	<i>Haemophilus</i> sp.	Human saliva	Kilian & Schiøtt (1975)
HK307, 313, 314, 316, 317, 320	<i>Haemophilus</i> sp.	Dental plaque	Kilian & Schiøtt (1975)
<i>Haemophilus parasuis</i>			
HK362	<i>H. suis</i>	Piglet, broncho-pneumonia	H. U. Bertschinger (538/70)†
HK417	<i>H. parasuis</i>	—	CCM5749
HK418	<i>H. parasuis</i>	—	CCM5751
HK422	<i>H. suis</i>	—	CIP52203

Study reference number	Received as	Isolated from	Source* and strain designation
<i>Haemophilus parasuis</i> (cont.)			
HK423	<i>H. suis</i>	—	CCM5747
HK424	<i>H. suis</i>	—	NCTC4557; cotype
HK425-427	<i>H. suis</i>	—	NCTC6359, NCTC7440 and NCTC7441, respectively
<i>Haemophilus pleuropneumoniae</i>			
HK351-355, 357-361	<i>H. parahaemolyticus</i>	Pig, pleuropneumonia	R. Nielsen (strains 4969, 2282, 2226, 5617, 5130, 2934, 2845, 4226, 2374 and 9711, respectively); all serotype 2
HK356	<i>H. parahaemolyticus</i>	Pig, pleuropneumonia	R. Nielsen (Canada); serotype 'K 17' (Schiefer <i>et al.</i> , 1974)
HK363	<i>Haemophilus</i> sp.	Pig, pneumonia	H. U. Bertschinger (543/70)†
HK405	<i>H. parahaemolyticus</i>	—	CCM5869; serotype 1
HK406	<i>H. parahaemolyticus</i>	—	CCM5870; serotype 2
HK407	<i>H. parahaemolyticus</i>	—	CCM5871; serotype 3
<i>Haemophilus paragallinarum</i>			
HK364	<i>H. paragallinarum</i>	—	E. L. Biberstein (H37)
HK381	<i>H. gallinarum</i>	Fowl, respiratory tract	NCTC3438
Unnamed taxon A			
HK164, 165, 167, 170	<i>Haemophilus</i> sp.	Conjunctivitis	N. Hegazy (Cairo)†
Unnamed taxon B			
HK366	<i>Haemophilus</i> sp.	Rat, lung	R. Nielsen
HK445, 446, 447	<i>Haemophilus</i> sp.	Rat, oral cavity	—
Unnamed taxon C			
HK382	<i>H. gallinarum</i>	—	P. H. A. Sneath (J102), CAPM5015§
HK383	<i>Haemophilus</i> sp.	Cock	P. H. A. Sneath (J103), CAPM5066§
HK384	<i>Haemophilus</i> sp.	Cock	P. H. A. Sneath (J104), CAPM5080§
HK428	<i>H. parainfluenzae</i> var. <i>suis</i>	Pig, pneumonia	P. H. A. Sneath (J94), CAPM5111§
HK429	<i>H. parasuis</i>	Pig, pneumonia	P. H. A. Sneath (J95), CAPM5113§
<i>Haemophilus piscium</i>			
HK419-421, 448-450	<i>H. piscium</i>	Trout ulcer	ATCC10801, ATCC14360, ATCC15711, ATCC14361, ATCC14362 and ATCC14363, respectively
<i>Actinobacillus actinomycetemcomitans</i>			
HK432	<i>A. actinomycetemcomitans</i>	—	W. Frederiksen (P202) (King 1153)
HK433	<i>A. actinomycetemcomitans</i>	—	W. Frederiksen (P228) (Holm 248/48-49)
HK434	<i>A. actinomycetemcomitans</i>	—	W. Frederiksen (P230) (Holm 363/49-50)
HK435	<i>A. actinomycetemcomitans</i>	Pus	W. Frederiksen (P506)
HK436	<i>A. actinomycetemcomitans</i>	Human blood	W. Frederiksen (200771)
HK442, 443	<i>A. actinomycetemcomitans</i>	—	NCTC9709 and NCTC9710, respectively

Study reference number	Received as	Isolated from	Source* and strain designation
<i>Eikenella corrodens</i>			
HK328	<i>H. aphrophilus</i>	—	J. Frazer (Turk)
HK340, 341	<i>H. canis</i>	Oral cavity	W. Sims (strains 18 and 35 respectively)
HK440	<i>Ei. corrodens</i>	—	J. Henriksen (Eiken 310A)
HK441	<i>Bacteroides corrodens</i>	—	NCTC10596
<i>Pasteurella multocida</i>			
HK437-439	<i>P. multocida</i>	—	W. Frederiksen (strains P437, P433 and P444, respectively)

* Abbreviations: ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A.; CAPM, Collection of Animal Pathogenic Microorganisms, Brno-Medlány, Czechoslovakia; CCM, Czechoslovak Collection of Microorganisms, Brno; CIP, Collection de l'Institut Pasteur, Paris, France; NCTC, National Collection of Type Cultures, Colindale, London; E. L. Biberstein, School of Veterinary Medicine, University of California, Davis, California, U.S.A.; P. Bülow, Department of Medical Bacteriology, Statens Seruminstitut, Aarhus Kommunehospital, Aarhus, Denmark; C. R. Dawson, Francis I. Proctor Foundation for Research in Ophthalmology, University of California, San Francisco, U.S.A.; J. Frazer, Department of Microbiology, School of Medicine, Leeds; W. Frederiksen, Department of Medical Bacteriology, Statens Seruminstitut, Aalborg Amtssygehus, Aalborg, Denmark; H. Lautrop, Department of Diagnostic Bacteriology, Statens Seruminstitut, Copenhagen, Denmark; C. H. Mordhorst, Ornithosis Department, Statens Seruminstitut, Copenhagen; R. Nielsen, Statens Veterinære Serumlaboratorium, Copenhagen; W. Sims, Royal Dental Hospital, London; P. H. A. Sneath, M.R.C. Microbial Systematics Unit, Leicester; K. Zinnemann, Department of Microbiology, School of Medicine, Leeds.

† Strains received from H. Lautrop.

‡ Strains isolated and partly described by Reymann (1951). Ampoules of the strains, freeze-dried in 1946-1949, were received from the Serum and Vaccine Department, Statens Seruminstitut, Copenhagen. See Results.

§ Strains described by Sneath & Johnson (1973).

Table 2. *Sites from which the 426 Haemophilus strains studied originated*

Origins	No. of strains
Strains from humans:	
Respiratory tract	83
Oral cavity	105
Meningitis	47
Blood cultures and/or epiglottitis	6
Otitis media	29
Respiratory infections	21
Conjunctivitis	43
Endocarditis	6
Chancroid	9
Vaginal and urethral cultures	8
Various other infections or origin unknown	22
Strains from animals:	
Swine	26
Fowls	5
Fish	6
Various other animals	10

Capsule production and serotyping of capsulated strains

Strains producing iridescent colonies on Levinthal's agar after 18 h incubation were typed by slide agglutination and the capsule swelling technique using antisera a to f (Hyland Laboratories, Los Angeles, U.S.A.).

Biochemical and physiological tests

Growth factor requirements. The requirement for V-factor was tested by observing the satellitism phenomenon on 10 % (v/v) citrate-stabilized calf blood agar streaked with a strain of *Staphylococcus aureus*. As a confirmatory test for selected strains, V-factor discs (Oxoid) were used on brain-heart agar (Difco) or calf blood agar. The ability to carry out the biosynthesis of porphyrins from δ -aminolaevulinic acid (ALA) was determined by the Porphyrin test (Kilian, 1974). Porphyrin was demonstrated by observation of fluorescence under Wood's light.

Requirement for increased CO₂ tension in the incubation atmosphere was determined by comparing growth on a lightly inoculated duplicate set of chocolate agar plates incubated in air and in an anaerobic jar containing air plus 10 % CO₂.

Haemolysis of horse blood around single colonies was examined after growth for 24 to 48 h on 5 % (v/v) defibrinated horse blood in blood agar base (Difco). A streak of *S. aureus* served as supply of V-factor.

Haemagglutination test. The method was essentially that used by Davis, Pittman & Griffiths (1950). Equal quantities (0.4 ml) of a 5 % (v/v) suspension of red blood cells of group O in saline (0.85 %, v/v, NaCl) and a 24 to 48 h Levinthal broth culture were mixed in a Perspex agglutination tray. The results were read from the pattern of the sedimented cells on the bottom of the depressions after incubation at room temperature for 2 h.

Reduction of nitrate and nitrite was tested as described by Cowan & Steel (1965) after 5 days growth in Levinthal broth with 0.1 and 0.01 % KNO₃, respectively. A Durham tube was inserted to detect gas production.

Indole production was demonstrated in a 24 to 48 h Levinthal broth culture and by the NCTC method (Clarke & Cowan, 1952) using Kovács's reagent.

Catalase activity. A few colonies were removed from a chocolate agar culture with a coverslip and placed in a drop of 3 % (v/v) H₂O₂ on a glass slide. Effervescence was noted.

Oxidase activity was tested by Kovács's method (1956).

Production of acid from carbohydrates was determined in phenol red broth base (Difco). The respective carbohydrates were added to the autoclaved and cooled medium as sterile 25 to 50 % (w/v) solutions to give a final concentration of 1 % (w/v). Haemin (BDH) dissolved in triethanolamine (Kodak) and NAD grade III (Sigma) were added in filter-sterilized solutions to a final concentration of 10 µg/ml. The broth (pH 7.6) was dispensed in 7 ml volumes in tubes, and a Durham tube was inserted in the glucose broth for the detection of gas. The tubes were inoculated with a loopful of bacteria and incubated for 5 days. The final pH was measured on a pH-meter (Radiometer, Copenhagen, Denmark). The following carbohydrates were used: D(+)-glucose, sucrose, 2-deoxy-D-ribose, D(+)-xylose, lactose, and D(−)-mannitol throughout the study, and fructose, D(+)-galactose, raffinose, inulin, dulcitol, D(−)-sorbitol, D(−)-arabinose and cellobiose with some of the strains. An inoculated control of basal medium without carbohydrate was included for each strain to detect any change in pH of non-glycolytic origin.

Hydrogen sulphide production was determined by placing a lead acetate strip (Merck) in the lid of an inoculated chocolate agar plate and reading after 2 days incubation. Distinct blackening of the paper was recorded as a positive result. In addition, a ferrous chloride medium was used. This contained 18.5 g brain-heart infusion (Difco), 6.0 g Noble agar (Difco), and 440 ml distilled water. After autoclaving and cooling to 50 °C, 2.5 ml of a 10 % (w/v) filter-sterilized aqueous solution of FeCl₂ was added. Haemin and NAD dissolved in 60 ml water were added to give a final concentration of 10 µg each/ml. The medium was

distributed in narrow tubes, inoculated with a platinum needle and read after incubation for 5 days.

DNAase activity was determined on the following agar medium: brain–heart infusion, 18.5 g; Noble agar, 6.0 g; calf thymus DNA (Sigma), 2 g; distilled water, 440 ml. After autoclaving and cooling to 50 °C, haemin and NAD were added as described above. Inoculated plates were incubated for 2 days, flooded with 1 M-HCl and examined for clear zones. A strain of *S. aureus* acted as a positive control.

Esterase activity was determined with indoxyl acetate (Koch–Light) discs placed on top of the growth on a chocolate agar plate as described by Kersters & De Ley (1971). A blue colour developing within 10 min was recorded as positive.

Micromethods. Determinations of the enzymes or enzyme systems were performed by suspending a loopful of bacteria from a 24 to 48 h chocolate agar culture in 0.5 ml (unless otherwise stated) of the respective enzyme substrates. The results were recorded after incubation for 4 and 48 h. Urease was tested as described by Lautrop (1960). Decarboxylation of ornithine and lysine and dihydrolation of arginine were determined in the media (1 ml) of Møller (1955). Alkaline phosphatase was determined as described by Kersters & De Ley (1971). Phenylalanine deaminase activity was determined in a modified Bergquist & Seracy (1963) medium. The enzyme substrate was 1 ml of a 0.5 % (w/v) aqueous solution of L-phenylalanine (Calbiochem). Aqueous ferric chloride solution (8 %, w/v) was used as the test reagent. The glycosidases, β -galactosidase, α -glucosidase, β -glucosidase, β -glucuronidase, β -xylosidase and α -fucosidase were determined in 0.1 % (w/v) solutions of the following chromogenic nitrophenyl glycosides: 2-nitrophenyl- β -D-galactopyranoside (Koch–Light), 4-nitrophenyl- α -D-glucopyranoside (Merck), 4-nitrophenyl- β -D-glucopyranoside (Merck), 4-nitrophenyl- β -D-glucopyranosiduronic acid (Merck), 2-nitrophenyl- β -D-xylopyranoside (Koch–Light), and 4-nitrophenyl- α -L-fucopyranoside (Koch–Light) (Kilian & Bülow, 1976).

Gas chromatography

The gas evolved during growth in glucose medium was analysed as follows: 500 ml of the glucose fermentation medium contained in an initially gas-free Erlenmeyer flask with arrangements for pressure adjustment, were inoculated and incubated for 48 h. The pH was then lowered to below 1 by addition of conc. HCl to drive off CO₂ dissolved in the medium. After 24 h, 300 μ l of the gas were collected and injected into a gas chromatograph equipped with a Gow–Mac thermal conductivity cell (w2 $\times$ filaments). The two columns were packed with Porapak Q (80 to 100 mesh) and molecular sieves (0.5 nm, 50 to 80 mesh), respectively. The carrier gas was helium, 50 ml/min. The determinations were carried out at 25 °C.

DNA extraction and purification

All strains were harvested from 24 to 48 h chocolate agar cultures and rechecked for identity. The bacteria were washed in cold saline–EDTA (0.15 M-NaCl–0.1 M-EDTA, pH 8), resuspended in saline–EDTA and lysed with sodium lauryl sulphate according to Marmur (1961). The DNA was extracted and purified by Stenderup & Bak's (1968) modification of Marmur's (1961) method. The final precipitate of pure DNA was dissolved in standard saline citrate (SSC; 0.15 M-NaCl–0.015 M-trisodium citrate, pH 7.0) and stored at 4 °C over a few drops of chloroform.

Determination of DNA base composition by thermal denaturation

The measurements were carried out on one of two different systems. One was that described by Stenderup & Bak (1968), except for the introduction of an electronically-heated cuvette holder and a Unicam SP 876 temperature controller in the arrangement. The temperature was increased by 0.25 °C/min, and the recorded temperature values were used without corrections. The alternative arrangement consisted of a Gilford spectrophotometer 2400 equipped with a thermosensor model 2417. The cuvettes were heated 0.5 °C/min by a Hetotherm gradient unit type PG which circulated ethylene glycol through the cuvette holder. The values for mean denaturation temperature (T_m) obtained from this apparatus were corrected by reference to a DNA preparation measured simultaneously. Three to four measurements were carried out on each DNA sample.

RESULTS

General survey of the taxonomic results

The 426 *Haemophilus* strains were assigned to species according to the results of the examination (Table 3). The results for each taxon are described in detail below. In nearly all cases it was possible to equate the preliminary taxa with existing named species, although type strains are available for only a few of these. However, several of the species pose different problems, mainly of nomenclature, and these are discussed below.

The two species *H. influenzae* and *H. parainfluenzae* have been subdivided into a number of biotypes. According to the results, *H. aegyptius*, *H. parahaemolyticus* and *H. paraphrohaemolyticus* do not merit specific status, and strains received with these labels have been included in *H. influenzae* or *H. parainfluenzae*. In Table 3, *H. aphrophilus* is placed after *H. paraphrophilus*. The similarity between the two species is significant, and they will be described together, although it is questionable whether *H. aphrophilus* belongs to the genus *Haemophilus*. *Haemophilus ducreyi* presents special problems. The nine strains assigned to this species divide naturally into two groups consisting of seven and two strains respectively. Only the latter two can legitimately be assigned to the genus *Haemophilus*, but as it is impossible to decide which of these groups are identical to Ducrey's bacillus (1889), all nine strains will be described under the heading *H. ducreyi*.

It has been possible to recognize four taxa among the strains not assignable to any of the previously described species. One of these, consisting of 17 strains mainly isolated from the human oral cavity, merits specific status and the name *Haemophilus segnis* is proposed. The three other taxa, also NAD-dependent and haemin-independent strains, are temporarily designated taxa A, B and C, respectively, because of the small number of strains available. The species *H. piscium* has been rejected from the genus *Haemophilus* for various reasons, but the strains will be described according to the label under which they were received.

Table 4 lists the phenotypic characters which are most useful for distinguishing the named species of *Haemophilus*. The characters included in the Table are those that seem to be constant and most reproducible in tests.

Observations on some of the methods and characters

Satellite phenomenon. Satellitism, which was used for the demonstration of V-factor requirement, could not always be demonstrated convincingly on horse blood agar, but calf blood agar nearly always gave satisfactory and clear-cut results. In case of doubt, a V-factor

Table 3. *Specific assignment of 426 Haemophilus strains*

Species	No. of strains	Species	No. of strains
<i>H. influenzae</i>		<i>H. paraphrophilus</i>	11
Biotype I	71	<i>H. aphrophilus</i> (<i>species incertae sedis</i>)	16
Biotype II	64	<i>H. segnis</i> sp. nov.	17
Biotype III	28	<i>H. parasuis</i>	9
Biotype IV	12	<i>H. pleuropneumoniae</i>	15
Biotype V	10	<i>H. paragallinarum</i>	2
Unclassified strains	3	Unnamed NAD-dependent taxa:	
<i>H. haemolyticus</i>	9	Taxon A	4
<i>H. haemoglobinophilus</i>	5	Taxon B	4
Unclassified strain	1	Taxon C	5
<i>H. ducreyi</i>	2	Strains rejected from <i>Haemophilus</i> :	
<i>H. parainfluenzae</i>		<i>H. piscium</i>	6
Biotype I	45	<i>H. ducreyi</i> ?	7
Biotype II	45		
Biotype III	31		
Unclassified strains	4		

requirement of haemin-independent strains could be verified on brain-heart agar supplied with a NAD-containing disc. Some strains received as *H. ducreyi*, but not assignable to the genus *Haemophilus*, showed a slight satellite growth around staphylococcal colonies on horse blood agar, but the same growth-enhancing effect could not be obtained around a NAD-containing disc.

Haemolysis. Several of the strains, primarily isolated as haemolytic, lost the property after one or more subcultures, with consequent problems in the classification of some of the NAD-dependent strains of human origin. The instability of haemolysis was also noted with strains assigned to the species *H. pleuropneumoniae*, but the species was fairly well defined by many other phenotypic traits.

Requirement for carbon dioxide. Nearly all strains assigned to the species *H. paraphrophilus*, *H. aphrophilus*, and the NAD-dependent taxon C grew better in air supplemented with 10 % CO₂. A CO₂ stimulation was also noted with a few strains assigned to other groups, but such strains did not differ otherwise from the remaining strains of the taxonomic groups to which they were assigned. *Haemophilus paraphrohaemolyticus* has therefore not been listed as a separate species.

Fermentation reactions and production of gas. A pilot study revealed that only a limited number of carbohydrates were of value for differentiating between species. However, glucose, sucrose, lactose, mannitol, xylose and deoxyribose were used throughout the study and proved useful. With a few exceptions, acid was produced in sufficient amounts to give a clear-cut change of indicator within 1 to 2 days' incubation. The pH normally decreased from 7.6 to between 5.0 and 6.3. The decrease of pH of non-glycolytic origin, as measured in the inoculated control tube, never exceeded 0.4 pH units. There was no rise in pH in the sugar-free medium with any of the strains after incubation for 5 days, but tubes occasionally showed an alkaline reaction after about 10 days.

Gas was evolved after 1 to 3 days in glucose broth by strains of the species *H. haemolyticus*, *H. parainfluenzae*, *H. paraphrophilus*, *H. aphrophilus* and *Actinobacillus actinomycetem-comitans*. The amount of gas produced was never very great (5 to 20 % of the total volume of the Durham tube) but was more pronounced with the last three species. Chromatographic studies of gas produced by representative aerogenic strains revealed that the constituents were hydrogen and carbon dioxide. Evolution of gas of the same composition was also seen in 0.01 % nitrate broth with most strains of the species mentioned, whereas only strains of

H. aphrophilus and *H. paraphrophilus* produced gas in 0.1 % nitrate broth. Nitrogen was not detected in any of the gas samples examined.

Reduction of nitrate and nitrite. All strains assigned to *Haemophilus*, except one unnamed strain, reduced nitrate. No significant differences were found between the levels to which nitrate was reduced by strains of the different taxa. Although most strains of the purely NAD-dependent species, in contrast to strains of *H. influenzae* and *H. haemolyticus*, were capable of reducing all nitrate beyond nitrite in 0.1 % nitrate broth, these two species were also capable of reducing beyond nitrite provided the amount of nitrate in the medium was reduced to 0.01 %.

Oxidase and catalase activity. All strains of *H. influenzae* were oxidase positive, although in some strains the reaction was delayed (15 s). Most other species gave equivocal results, and only strains of *H. aphrophilus* and *H. ducreyi* were invariably devoid of oxidase activity. All strains were oxidase negative when picked from Levinthal's agar. Similarly, with few exceptions, strains of *H. influenzae*, *H. haemolyticus*, biotype III of *H. parainfluenzae*, *H. parasuis* and *H. paragallinarum* were strong catalase producers. Strains of the species *H. paraphrophilus*, *H. aphrophilus* and *H. ducreyi* were catalase negative, whereas the remaining species gave equivocal results.

Hydrogen sulphide production. Hydrogen sulphide was never detected in the ferrous chloride medium, whereas lead acetate paper divided the strains into those causing no change and those giving a distinct blackening within 2 days.

DNAase activity. DNAase activity was not observed.

Esterase activity. In the esterase test, results ranged from a strong blue colour developed within 1 min to complete absence of a reaction. Esterase activity was encountered in some haemolytic strains assigned to *H. parainfluenzae*, and some strains of the species *H. paraphrophilus*, *H. aphrophilus*, *H. pleuropneumoniae*, *H. parasuis* and *H. paragallinarum*. Because of poor reproducibility, the character has not been included in the Tables.

Micromethods. The micromethods used for the detection of porphyrin synthesis, production of indole, urease, amino acid decarboxylases, glycosidases and alkaline phosphatase proved satisfactory for the examination of nearly all strains. Only some *H. parasuis* strains growing with diminutive colonies on chocolate agar presented difficulties in obtaining sufficiently large inocula. The same problem also applied to strains of *Eikenella corrodens* producing colonies adhering to the agar plate. With such strains, satisfactory tests could be performed with bacteria harvested from a broth culture by centrifugation.

The media used for determination of decarboxylases were originally devised for growth (Møller, 1955) but, although not meeting the growth requirements of haemophili, the results obtained with a heavy suspension of bacteria were identical to those obtained with the same media supplemented with NAD and haemin. Arginine dihydrolase was demonstrated only in occasional strains.

All *Haemophilus* strains, except for strains of *H. haemoglobinophilus* and a strain of *H. parainfluenzae* isolated from a rat, produced alkaline phosphatase.

The micromethods used normally gave strong and clear-cut results within 4 h incubation.

DNA base composition. Seventy-nine strains were analysed for the guanine + cytosine (GC) content of their DNA (Table 5). *Escherichia coli* B was included for reference purposes. There was good agreement between results obtained with the two systems used to determine T_m , with results varying by only ± 0.2 °C from the mean. Table 5 gives the mean values for each species and the calculated standard deviations, excluding unclassified strains. Figure 1 summarizes graphically the mean GC content of the DNA of *Haemophilus* species and the biotypes of species. The range of values obtained for strains assigned to the genus

Table 5. GC content of DNA of species of *Haemophilus* and related genera

Species	Biotype	No. of strains tested*	Mean GC (mol %, $\pm$ S.D.)	
			Biotype	Species
<i>H. influenzae</i>	I	4	38.5 $\pm$ 0.12	38.7 $\pm$ 0.44
	II	4	39.1 $\pm$ 0.31	
	III	5	38.6 $\pm$ 0.28	
	IV	4	38.9 $\pm$ 0.48	
	V	2	38.3 $\pm$ 0.71	
	Unc.	1	38.3	
<i>H. haemolyticus</i>	—	3	—	38.5 $\pm$ 0.0
<i>H. haemoglobinophilus</i>	—	3	—	38.1 $\pm$ 0.96
	Unc.	1	37.6	
<i>H. ducreyi</i>	—	2	—	37.8 $\pm$ 0.14
<i>H. parainfluenzae</i>	I	5	39.7 $\pm$ 0.22	40.2 $\pm$ 0.51
	II	7	40.2 $\pm$ 0.35	
	III	8	40.6 $\pm$ 0.49	
	Unc.	1	43.7	
<i>H. aphrophilus</i>	—	5	—	42.0 $\pm$ 0.58
<i>H. paraphrophilus</i>	—	5	—	41.9 $\pm$ 0.69
<i>H. segnis</i>	—	4	—	43.5 $\pm$ 0.54
<i>H. parasuis</i>	—	1	—	41.5
<i>H. pleuropneumoniae</i>	—	3	—	42.0 $\pm$ 0.35
<i>H. paragallinarum</i>	—	1	—	42.2
Taxon A	—	1	—	39.3
Taxon C	—	2	—	42.0 $\pm$ 0.0
<i>H. piscium</i>	—	2	—	55.1 $\pm$ 0.0
<i>A. actinomycetemcomitans</i>	—	1	—	42.7
<i>Ei. corrodens</i>	—	1	—	55.9
<i>E. coli</i> B	—	1	—	51.7

Unc., unclassified strain.

* For strains tested, see text.

Haemophilus was about 37 to 44 mol %. All haemin-requiring species had a GC content of DNA below 39.4 mol %, whereas all haemin-independent species had a GC content above this value. The difference between the two main groups is significant at the 0.1 % level (Table 6). Strains assigned to *H. segnis* had the highest GC value.

The calculated significant differences of mean GC contents (sum of squares simultaneous test, SS-STP; Sokal & Rohlf, 1969) are provided in Table 6.

Characteristics of the individual taxa

The taxa to be described must be considered preliminary at this stage because type strains are available only for a few of the *Haemophilus* species. The names used are those used in the revised classification resulting from this study. Although this means that the main results of the study are anticipated it makes the presentation easier to follow. Most of the taxonomic deliberations and decisions are presented under Discussion.

Haemophilus influenzae

One hundred and eighty-eight strains isolated from humans were assigned to *H. influenzae* (Table 1).

Fig. 1. The GC contents of the DNAs of 11 *Haemophilus* species. Values plotted are the means $\pm$ 1 S.D.

Characteristics of H. influenzae. The strains assigned to *H. influenzae* included all non-haemolytic strains that were V-factor-dependent and devoid of the enzymic capacities to convert δ -aminolaevulinic acid to porphyrins. A number of almost constant properties further characterized the strains of the species. They were small, non-motile, slightly pleomorphic rods or coccobacilli with only occasional filamentous forms. Colonies on chocolate agar were smooth, low, convex, greyish, translucent and attained a diameter of 0.5 to 1.0 mm. Capsulated strains usually produced larger and more mucoid colonies (1 to 3 mm) and showed a greater tendency to coalesce with no visible line of division than colonies of non-capsulated strains. Carbohydrates were fermented to a final pH of 5.3 to 5.9. All strains fermented glucose and deoxyribose with the production of acid but no gas. Most strains fermented xylose but none fermented sucrose, lactose or mannitol. With a few exceptions they were catalase and oxidase positive, possessed alkaline phosphatase and were H_2S and esterase negative. All strains were devoid of α -glucosidase, β -glucosidase, β -galactosidase,

Table 6. Significant differences of mean GC contents of DNAs of *Haemophilus* species

<i>H. influenzae</i>										
<i>H. haemolyticus</i>	NS									
<i>H. haemoglobinophilus</i>	NS	NS								
<i>H. ducreyi</i>	NS	NS	NS							
<i>H. parainfluenzae</i>	***	**	***	***						
<i>H. paraphrophilus</i>	***	***	***	***	***					
<i>H. aphrophilus</i>	***	***	***	***	***	NS				
<i>H. segnis</i>	***	***	***	***	***	*	NS			
<i>H. parasuis</i>	**	**	**	**	NS	NS	NS	NS		
<i>H. pleuropneumoniae</i>	***	***	***	***	**	NS	NS	NS	NS	
<i>H. paragallinarum</i>	***	***	***	***	NS	NS	NS	NS	NS	NS
	<i>H. influenzae</i>	<i>H. haemolyticus</i>	<i>H. haemoglobinophilus</i>	<i>H. ducreyi</i>	<i>H. parainfluenzae</i>	<i>H. paraphrophilus</i>	<i>H. aphrophilus</i>	<i>H. segnis</i>	<i>H. parasuis</i>	<i>H. pleuropneumoniae</i>

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.

β -xylosidase, β -glucuronidase (except for HK224, 235, 387 and 388) and α -fucosidase, and none of the strains required CO₂ for good growth. The GC content of their DNA (mean value for the 19 strains HK6, 30, 32, 76, 223, 238, 279, 281, 367, 369, 387, 388, 389, 390, 393, 396, 397, 400 and 401) was 38.7 ± 0.44 mol % (Table 5).

Sixty-eight (36%) of the strains were capsulated. All but two could be assigned to one of Pittman's serotypes a to f. The proportion of capsulated strains and the distribution of six serotypes are summarized in Table 8.

Subdivision of H. influenzae. All except three of the strains included in the species *H. influenzae* could be subdivided into five biotypes (I to V). Strains received as *H. aegyptius* were assigned to biotype III. The differential characteristics of the biotypes are summarized in Table 7, and the relationship between the biotypes and the sites from which they were isolated is shown in Table 18, below.

Biotype I. Seventy-one strains belonged to this biotype (Table 1). With a few exceptions the strains were isolated from acute infections only. Growth of the strains gave a pungent smell of indole. All strains were indole, urease, and ornithine decarboxylase positive. Seventy-three per cent of the strains were capsulated, and serotype b was predominant (Table 8). The phenotypic characters of the strains are given in Table 7. Two strains (HK141, HK293) did not grow in the fermentation media.

Biotype II. Sixty-four strains were included in this biotype (Table 1). The lack of ornithine decarboxylase was the most prominent phenotypic character distinguishing biotype II from biotype I. The strains were isolated from various infections as well as from the flora of normal throats. Growth on agar media produced the same characteristic smell of indole as for strains of biotype I. Thirteen per cent of the strains were capsulated, but one of them was not typable with Pittman type antisera a to f (Table 8). The phenotypic characters of the strains are given in Table 7.

Biotype III. The 28 strains of biotype III (Table 1) were, with a few exceptions, isolated from cases of conjunctivitis and from normal throats. Some of the strains produced long

Table 7. *Haemophilus influenzae*, characteristics which serve to differentiate five biotypes among 185 strains

None of the strains formed gas in glucose broth or fermented sucrose, lactose or mannitol. None produced β -galactosidase, α -glucosidase, β -glucosidase, β -xylosidase or α -fucosidase. All were negative in the Porphyrin test and none required CO₂.

	No. of positive strains					Negative strains*
	Biotype I (71 strains)	Biotype II (64 strains)	Biotype III (28 strains)	Biotype IV (12 strains)	Biotype V (10 strains)	
Indole	71	64	0	0	10	All except HK54, 207, 219, 223, 232, 236, 237, 240, 242, 243, 246, 248, 399, 400 All except HK228
Urease	71	64	28	12	0	
Ornithine decarboxylase	71	0	0	12	10	
Lysine decarboxylase	3	6	1	2	2	
Arginine dihydrolase	0	1	0	0	0	All except HK17, 32, 394; weak: HK171, 234, 236, 343, 390, 398, 400, 401
H ₂ S (lead acetate)	4	0	0	7	0	
Glucose, acid	69	64	28	12	10	All except HK224, 235, 387, 388 HK32, 209; weak: HK400 HK343, 399, 400; weak: HK390, 393 All except HK264, 265, 269, 270, 277, 279, 286, 291, 292, 294, 367, 368, 369 See Table 8
Xylose, acid	65	50	22	11	10	
Deoxyribose, acid	69	64	28	12	10	
β -Glucuronidase	2	0	2	0	0	
Catalase	71	64	28	10	10	All except HK264, 265, 269, 270, 277, 279, 286, 291, 292, 294, 367, 368, 369 See Table 8
Oxidase	71	64	28	9	10	
Haemagglutination	0	1	12	0	0	
Capsule	52	8	0	7	0	

* Only exceptions within the individual biotypes are listed.

Table 8. *Proportion of 188 H. influenzae strains which formed capsules*

	Total no. of strains	No. of capsulated strains	Serotype, and no. of capsulated strains	Reference nos. of capsulated strains
Biotype I	71	52 (73 %)	a: 3 b: 45 f: 4	HK190, 390, 391 HK27, 76, 162, 163, 171, 173-189, 191, 193-205, 213-216, 254, 293, 297, 392, 393 HK172, 192, 247, 403
Biotype II	64	8 (12 %)	b: 4 c: 2 f: 1 NT: 1	HK206, 210, 211, 212 HK342, 396 HK402 HK168
Biotype III	28	0	—	—
Biotype IV	12	7 (58 %)	b: 2 d: 2 e: 3	HK209, 394 HK343, 397 HK399, 400, 401
Biotype V	10	0	—	—
Unclassified	3	1 (33 %)	NT	HK280

NT, Not typable with antisera a to f.

slender rods in addition to the normal microscopic picture of *H. influenzae*, but the relative number of these rods varied considerably from strain to strain. This property was most pronounced in HK367 and HK368. Two strains (HK71 and HK72) showed a markedly pleomorphic microscopic picture, with long irregular filaments. Colonies formed by strains of biotype III were generally smaller than those of the other biotypes of *H. influenzae*, and the odour emitted by them was sweet. None of the strains were capsulated. Forty-four per cent of the strains agglutinated red blood cells, but three had lost the property when retested. Only strains isolated from conjunctivitis were haemagglutinating. The phenotypic characters of the strains are shown in Table 7.

Biotype IV. Twelve strains were included in biotype IV (Table 1), which was the least satisfactory biotype of *H. influenzae*. Several of the common characters of the species were variable within biotype IV. Six of the 12 strains were received from culture collections as representatives of serotypes d and e of *H. influenzae* (HK398 is a converted rough strain of type d) (Table 8). The odour emitted by strains of this biotype was sweet. The phenotypic characters of the strains are given in Table 7.

Biotype V. All ten strains of this biotype (Table 1) were isolated from ear infections, and the group was homogeneous. None of the strains was capsulated. Their characteristics are shown in Table 7.

Unclassified strains. The three remaining strains (Table 1), that could not be accommodated in any of the five biotypes, were very similar and might represent a sixth biotype. The strains were ornithine decarboxylase, catalase and oxidase positive, but indole, urease, lysine decarboxylase and arginine dihydrolase negative. They fermented glucose and xylose, and two of them (HK39 and HK281) fermented deoxyribose. None of the strains fermented sucrose, lactose or mannitol. One of the strains (HK280) was capsulated, but did not belong to the serotypes a to f. The DNA base composition as determined for one of the strains (HK281) was not significantly different from that of other strains of *H. influenzae* (Table 5).

Table 9. *Characteristics of 9 strains of H. haemolyticus*

All strains were negative in ornithine and lysine decarboxylases, arginine dihydrolase and glycosidases, and in the Porphyrin test.

	No. of positive strains	Negative strains
Indole	8	HK386
Urease	9	—
H ₂ S (lead acetate)	9	—
Haemolysis	9	See text
Glucose, acid	9	—
Glucose, gas	8	HK29
Sucrose, acid	0	—
Lactose, acid	0	—
Xylose, acid	5	HK33, 53, 75, 386
Deoxyribose, acid	9	—
Mannitol, acid	0	—
Catalase	9	—
Oxidase	9	—
CO ₂ improves growth	0	—
Haemagglutination	1	All except HK45

Haemophilus haemolyticus

Nine strains were included in this species (Table 1). These were all haemolytic strains that required V-factor and were unable to synthesize porphyrins from δ -aminolaevulinic acid. Apart from the haemolytic ability, these strains differed from strains of *H. influenzae* in producing gas from glucose broth and being H₂S positive. The other features of the group were very similar to those described for *H. influenzae*. They were small, regular, non-motile and non-capsulated coccobacilli or short rods with occasional filamentous forms. Colonies on chocolate agar were smooth, convex, greyish, translucent, and reached a diameter of 0.5 to 1.5 mm. Growth was not enhanced by excess of CO₂ in the incubation atmosphere. The indole-producing strains emitted a pungent smell. On primary isolation all strains were haemolytic, but one strain (HK12) lost the property after several transfers. Carbohydrates were fermented to a final pH of 5.3 to 5.7. The GC content of their DNA (mean value for the three strains HK53, HK75 and HK386) was 38.5 mol % (Table 5). The characteristics of the strains are summarized in Table 9.

Haemophilus haemoglobinophilus (canis)

Five strains isolated from dogs were assigned to this species (Table 1). In addition, an unclassified strain isolated from a human perianal abscess (Ryan, 1968) is also described here.

Characteristics of H. haemoglobinophilus. The strains included in *H. haemoglobinophilus* were fairly well distinguished from all other *Haemophilus* species by the requirement for haemin only, the production of indole and the fermentative pattern. They were also differentiated from all other haemophili studied by the failure to produce alkaline phosphatase. The following properties further characterized the strains of this species. They were small, slightly pleomorphic, non-motile and non-capsulated rods. Colonies on chocolate agar were smooth, convex and translucent with a small granular area on top, and reached a diameter of 1 to 2 mm. They grew well and without haemolysis on blood agar and showed no satellite growth around a streak of staphylococcus. Excess of CO₂ in the incubation atmosphere did not improve growth. The GC content of their DNA (mean value for the three strains HK365,

Table 10. *Biochemical and physiological characteristics of H. haemoglobinophilus and an unnamed strain*

All strains were non-haemolytic and negative in the Porphyrin test, and in urease, ornithine and lysine decarboxylases, and arginine dihydrolase.

	No. of positive strains		Negative strains
	<i>H. haemoglobinophilus</i> (5 strains)	NCTC10555/ HK431	
Indole	4	0	HK378 (see comment)
H ₂ S (lead acetate)	3	0	HK375, 378 (reactions weak)
Glucose, acid	5	1	—
Glucose, gas	0	0	—
Sucrose, acid	5	1	—
Lactose, acid	0	1	—
Xylose, acid	5	1	—
Deoxyribose, acid	4	1	HK376
Mannitol, acid	5	0	—
β -Galactosidase	2	1	HK365, 375, 378
α -Glucosidase	0	1	—
β -Glucosidase	0	1	—
β -Glucuronidase	0	0	—
β -Xylosidase	0	1	—
α -Fucosidase	0	0	—
Catalase	5	0	—
Oxidase	5	0	—
CO ₂ improves growth	0	0	—
Nitrate reduction	5	0	—
Alkaline phosphatase	0	1	—

HK376 and HK378) was 38.1 ± 0.96 mol % (Table 5). The phenotypic characters are listed in Table 10.

Unnamed strain. Strain HK431 has been considered as a variety of *H. influenzaemurium* (Ryan, 1968), but in the absence of identical or similar strains it is conveniently described under *H. haemoglobinophilus* because it resembles this species in several ways, especially in the requirement for haemin only. It was a non-capsulated coccobacillus with occasional filamentous forms. Colonies on chocolate agar were smooth, convex, greyish, translucent and reached a diameter of about 1 mm. Colonies on blood agar were smaller and there was no satellite effect of a staphylococcus on the plate. It was non-haemolytic and excess of CO₂ in the incubation atmosphere did not affect growth. The GC content of its DNA was 37.6 mol % (Table 5). The characteristics of the strain are listed in Table 10.

Haemophilus ducreyi

Nine strains designated *H. ducreyi* were included in this study (Table 1). Although only two of the strains can legitimately be assigned to the genus *Haemophilus*, they are all described here for the reasons discussed below.

Characteristics of the strains. Common to all nine strains was a very poor growth on all media. One of the strains (HK350) was distinctly Gram-positive and reduced nitrate, and since none of these characters agree with the description of Reymann (1949*b*), the authenticity of the strain is dubious and it will not be dealt with further. The remaining eight strains fell within two distinct groups, made up of the Reymann strains and the CIP strains respectively. The two groups will therefore be described separately.

Table 11. *Characteristics of two groups of strains designated H. ducreyi*

All strains were non-haemolytic, and indole, urease, ornithine and lysine decarboxylases, arginine dihydrolase, catalase and oxidase negative. None produced glycosidases.

	No. of positive strains		Negative strains
	Reymann's strains (6 strains)	CIP strains (2 strains)	
Porphyrin test	6	0	—
H ₂ S (lead acetate)	0	0	—
Glucose, acid	5*	0	HK349
Glucose, gas	0	0	—
Sucrose, acid	4*	0	HK348, 349
Lactose, acid	0	0	—
Deoxyribose, acid	0	0	—
Xylose, acid	0	0	—
CO ₂ improves growth	0	0	—
Nitrate reduction	0	2	—
Alkaline phosphatase	0	2	—

* Positive after incubation for 6 to 8 days.

Reymann's strains. All six strains (HK344 to HK349) were non-motile coccobacilli or short slender rods. They were predominantly Gram-negative but interspersed with varying numbers of cells that resisted total decolorization with ethanol. A closer examination by electron microscopy showed that the fine structure of the cell wall of these strains was typical of a Gram-positive bacterium (Kilian & Theilade, 1975). Colonies on chocolate agar were smooth, convex, greyish and translucent and reached a diameter of about 0.5 mm. Only very sparse growth was obtained on horse blood agar. There was slight satellite growth around a staphylococcus streak but not around a V-factor disc. The final pH in fermentation media after 6 to 8 days was 4.5 to 4.9. All attempts to extract DNA from the strains were unsuccessful. The characteristics of the strains are listed in Table 11.

CIP strains. These two strains (HK379 and HK380) were non-motile, irregular, slender rods often arranged in long chains. They were distinctly Gram-negative as shown by staining and by electron microscopic examination of the cell wall (Kilian & Theilade, 1975). Colonies on chocolate agar after 72 h incubation were predominantly small (about 0.5 mm), flat, smooth, greyish and translucent but with a few interspersed larger colonies (1.5 to 2.0 mm) with an otherwise identical appearance. Growth on horse blood was sparse and no haemolysis was seen. There was no satellitism around a staphylococcus streak on blood agar, and CO₂ had no effect on the growth. The GC content of their DNA (mean value for the two strains) was 37.8 ± 0.14 mol % (Table 5).

Haemophilus parainfluenzae

The 125 strains assigned to this species (Table 1) were mainly isolated from the oral cavities and throats of healthy persons.

Characteristics of H. parainfluenzae. The taxonomically important characters distinguishing *H. parainfluenzae* from *H. influenzae* were the enzymic capacities to carry out porphyrin synthesis, the ability to ferment sucrose and the inability to ferment deoxyribose and xylose. Among other phenotypic characters, fermentation reactions distinguished *H. parainfluenzae* from other haemin-independent *Haemophilus* species. The following properties characterized the species. They were small, non-motile, pleomorphic rods, normally with long filamentous forms. When grown on chocolate agar they emitted a sweet smell. Colonial

Table 12. *Biochemical and physiological characteristics of 125 strains of H. parainfluenzae*

None of the strains fermented lactose or xylose. All were positive in the Porphyrin test.

	No. of positive strains				Negative strains*
	Unclassified strains				
	Biotype I (45 strains)	Biotype II (45 strains)	Biotype III (31 strains)	(3 strains) (NCTC4101/HK408)	
Indole	0	0	0	0	—
Urease	0	45	31	2	HK147
Ornithine decarboxylase	45	45	0	2	HK15
Lysine decarboxylase	4	0	0	0	All except HK88, 116, 146, 151
Arginine dihydrolase	0	0	0	0	—
H ₂ S (lead acetate)	45	45	31	3	—
Haemolysis	0	16	20	0	All except HK3, 8, 19, 23, 26, 31, 36, 40, 48, 51, 52, 60, 64, 67, 117, 121, 152, 261, 303, 304, 312, 330–339, 385, 404, 411, 412, 413
Glucose, acid	45	45	31	3	Weak: HK16, 22, 26, 99, 105, 133
Glucose, gas	40	41	0	3	HK16, 34, 86, 99, 105, 124, 133, 282, 336
Fructose, acid	44	44	31	3	HK124, 138
Sucrose, acid	44	43	31	3	HK26, 142, 155; weak: 16, 86, 99, 105, 133
Maltose, acid	44	42	31	3	HK16, 26, 99, 105
Deoxyribose, acid	0	0	0	0	—
Sorbitol, acid	0	0	0	0	—
Mannitol, acid	0	0	0	0	—
Inulin, acid	0	0	0	0	—
β -Galactosidase	43	38	17	3	HK19, 23, 48, 49, 74, 107, 112, 122, 124, 137, 169, 255, 258, 260, 261, 276, 282, 303, 333, 334, 385, 404, 410
α -Glucosidase	0	0	0	0	—
Catalase	26	36	31	1	HK14, 15, 26, 40, 47, 77, 78, 80, 82, 86, 90, 91, 92, 94, 98, 100, 101, 109, 113, 115, 125, 130, 133, 139, 140, 155, 256, 257, 259
Oxidase	44	45	29	3	HK91, 411, 412; reaction often weak
Haemagglutination	1	0	4	1	All except HK48, 80, 169, 258, 276, 409
CO ₂ improves growth	4	0	12	0	All except HK19, 22, 51, 89, 94, 112, 118, 121, 128, 137, 330, 333, 411, 412, 413, 444

* Only exceptions within the individual biotypes are listed.

Table 13. *Characteristics of traditional species H. parahaemolyticus, H. paraphrohaemolyticus and two biotypes of H. parainfluenzae*

	No. of positive strains					
	<i>H. parainfluenzae</i>		<i>H. parahaemolyticus</i>			<i>H. paraphrohaemolyticus</i>
	Biotype II (29 strains)	Biotype III (11 strains)	'Biotype II' (16 strains)	'Biotype III' (12 strains)	Porcine strains* (15 strains)	(8 strains)
Haemolysis	0	0	16	12	15	8
Indole	0	0	0	0	0	0
Urease	29	11	16	12	15	8
Ornithine decarboxylase	29	0	16	0	0	0
Lysine decarboxylase	0	0	0	0	0	0
Arginine dihydrolase	0	0	0	0	0	0
H ₂ S (lead acetate)	29	11	16	12	15	8
Glucose, acid	29	11	16	12	15	8
Glucose, gas	26	0	15	0	0	0
Fructose, acid	28	11	16	12	15	8
Sucrose, acid	28	11	15	12	15	8
Lactose, acid	0	0	0	0	0	0
Xylose, acid	0	0	0	0	15	0
Deoxyribose, acid	0	0	0	0	15	0
Mannitol, acid	0	0	0	0	15	0
β -Galactosidase	23	5	15	6	15	6
Catalase	22	11	15	12	9	8
Oxidase (see text)	29	11	15	12	5	6
CO ₂ improves growth	0	4	0	0	0	8
Capsule	1	0	0	0	15	0

* Referred to as *H. pleuropneumoniae* in text.

morphology was variable and is discussed for each biotype. The strains were V-factor-dependent, indole negative, H₂S and alkaline phosphatase positive (except HK444), and fermented glucose, sucrose, maltose, galactose, and fructose but never lactose, deoxyribose, xylose, mannitol, dulcitol, sorbitol or inulin. Most strains produced β -galactosidase but none produced α -glucosidase, β -glucosidase, β -xylosidase or α -fucosidase (except HK413). Some of the strains were β -haemolytic and some required CO₂ for good growth. All were oxidase positive, although the reaction was weak in some, but the catalase reaction was variable. Only occasional strains were capsulated or haemagglutinating. The GC content of their DNA (mean value for the 20 strains HK3, 22, 23, 51, 65, 81, 90, 102, 124, 169, 282, 331, 335, 385, 404, 409, 410, 411, 412 and 416) was 40.2 ± 0.51 mol % (Table 5).

Subdivision of H. parainfluenzae. Except for four, the 125 strains which were assigned to *H. parainfluenzae* could be subdivided into three biotypes (I, II and III). The differential characteristics of the three biotypes are given in Table 12. Biotypes II and III, characterized by urease production, include haemolytic (*H. parahaemolyticus*) as well as non-haemolytic strains. Some of the haemolytic strains of biotype III showed improved growth when incubated with extra CO₂ (*H. paraphrohaemolyticus*). The characteristics of the haemolytic and non-haemolytic strains of biotypes II and III are listed separately in Table 13. Also included in the Table are the characteristics of haemolytic strains of porcine origin.

Biotype I. This biotype, which included 45 strains (Table 1), was the most homogeneous of the three biotypes of *H. parainfluenzae*. It comprised, with few exceptions, strains isolated from human saliva only. Colonies on chocolate agar were greyish-white or yellowish opaque and reached a diameter of 1 to 2 mm. On primary isolation, 50 % of the strains formed

smooth, convex colonies with an entire edge, 30 % very rough wrinkled colonies and 20 % formed flat smooth colonies with a serrated edge. Colonies of the two latter types were dry and coherent in texture and could often be slid intact across the surface of the agar plate. However, the colonial morphology was not a consistent property, as most of the strains of the rough type converted to the smooth type after some transfers. None of the strains was capsulated. The biochemical and physiological characteristics of the strains are listed in Table 12. With a few exceptions, the carbohydrates attacked were fermented to a final pH of 5.3 to 5.7.

Biotype II. The 45 strains of biotype II (Table 1) differed from those of biotype I in being urease positive. Sixteen strains (36 %) were β -haemolytic at primary testing but two of the strains (HK64 and HK336) had lost the property when retested. Two strains (HK120 and HK122) produced greening of horse blood agar. On chocolate agar the colonial morphology was of the same types as biotype I. The detailed phenotypic traits are listed in Table 12. With a few exceptions, carbohydrates were fermented to a final pH of 5.3 to 5.7.

Biotype III. The 31 strains of biotype III (Table 1) differed from the two previous biotypes, being ornithine decarboxylase negative, not producing gas from glucose, and giving a weaker acid production in carbohydrate media (final pH 5.6 to 7.0). Twenty of the strains (65 %) were haemolytic at primary isolation or on receipt. When retested, three strains (HK121, HK261 and HK338) had lost the property. Colonies on chocolate agar were butyrous, flat, greyish, smooth, translucent and reached a diameter of 0.5 to 1.5 mm. The growth of twelve strains (39 %) was enhanced by 10 % CO₂ added to the incubation atmosphere. The phenotypic characters of the strains are listed in Table 12.

Unclassified strains. The four remaining strains tentatively assigned to *H. parainfluenzae* shared most of the characters of this species. However, three of the strains (HK15, HK80 and HK147) differed by being indole positive. The other characters of the three strains are listed in Table 12. The fourth strain (HK408) occupied a unique position among all the strains of the collection. On superficial examination the strain was very similar to the strains of *H. parainfluenzae* biotype I. It had, however, a number of distinctive characters (Table 12), including a wider spectrum of carbohydrates attacked. Colonies on chocolate agar were smooth, convex, butyrous, reddish-translucent, and reached a diameter of 1.5 to 2.0 mm. The GC content of its DNA (43.7 mol %) was significantly different ($P < 0.001$) from the value obtained for other strains of *H. parainfluenzae* (Table 5).

The aphrophilus group

The two species *H. paraphrophilus* and *H. aphrophilus* were included in this group, to which 27 strains were assigned (Table 1).

Characteristics of the aphrophilus group. The group consisted of haemophili which almost all required CO₂ and which had nearly uniform characteristics and differed from all other haemophili in several phenotypic features. The specific characters of the group were the ability to ferment lactose, the fermentation of carbohydrates to a final pH of 4.9 to 5.4 and with production of relatively large amounts of gas from glucose, the α -glucosidase activity, and the requirement for CO₂ by most strains. The strains were non-motile, short regular rods with only occasional filamentous forms. Colonies on chocolate agar were highly convex, granular, yellowish, opaque and reached a diameter of 1.0 to 1.5 mm. When incubated without CO₂ the growth was characteristically stunted, with very small colonies interspersed with a few larger colonies. The odour of the growth was sweetish. Haemolysis was not seen on horse blood agar. Growth in broth was granular, with a heavy sediment on the bottom of the tube and adhering colonies on the walls. It was difficult to remove this

Table 14. *Biochemical and physiological characteristics of H. paraphrophilus, H. aphrophilus and Actinobacillus actinomycetemcomitans*

All strains were negative in indole, urease, ornithine and lysine decarboxylases, arginine dihydrolase, haemolysis, haemagglutination, β -glucuronidase, β -xylosidase and α -fucosidase. All strains were positive in alkaline phosphatase and the Porphyrin test.

	No. of positive strains			Negative strains*
	<i>H. paraphrophilus</i> (11 strains)	<i>H. aphrophilus</i> (16 strains)	<i>A. actinomycetem-</i> <i>comitans</i> (7 strains)	
V-factor requirement	11	0	0	—
H ₂ S (lead acetate)	11	0	0	—
Glucose, acid	11	16	7	—
Glucose, gas	10	15	5	HK296, 327, 434, 442
Fructose, acid	11	16	7	—
Sucrose, acid	11	16	0	—
Lactose, acid	11	16	0	—
Maltose, acid	11	16	7	—
Xylose, acid	1	0	2	All except HK414, 434, 442
Deoxyribose, acid	11	16	7	—
Mannitol, acid	0	0	7	—
β -Galactosidase	11	16	0	—
α -Glucosidase	9	14	0	HK85, 321, 325, 326
β -Glucosidase	1	0	0	HK415
Catalase	1	1	7	All except HK296, 327
Oxidase	11	0	0	—
CO ₂ improves growth	8	14	7	HK83, 85, 159, 325, 326

* Only exceptions within the individual species are listed.

adherent growth from the tube. It resembled the growth of *Streptococcus mutans* and *S. sanguis*, but in contrast to these bacteria the characteristic adherent growth was independent of the presence of sucrose in the medium. The GC content of their DNA (mean value for the ten strains HK83, 85, 159, 308, 325, 371, 372, 373, 414 and 415) was 41.9 ± 0.60 mol % (Table 5). The biochemical and physiological characters of *H. paraphrophilus* and *H. aphrophilus* are listed in Table 14.

Two strains (HK296 and HK327) were only tentatively assigned to the group, because they differed from all other strains in important ways: they were catalase positive, were anaerogenic in glucose broth, and failed to grow on chocolate agar with bacitracin (300 μ g/ml). Furthermore, HK296 produced very small colonies, even when incubated in air plus 10% CO₂.

The characters distinguishing these two species from *H. haemoglobinophilus* and from *Actinobacillus actinomycetemcomitans*, *Eikenella corrodens* and *Pasteurella multocida*, are summarized in Table 17.

Haemophilus segnis sp. nov.

The name *Haemophilus segnis* (from the Latin adjective *segnis*, sluggish) is proposed for a distinctive and not previously described group of V-factor-dependent and haemin-independent haemophili. The differentiation of the species from other haemophili is unfortunately based mainly on negative characters. The high GC content of the DNA, however, separates the strains from other species. Seventeen strains were assigned to the species (Table 1).

Characteristics of H. segnis. All strains were non-motile and non-capsulated, and were

Table 15. *Biochemical and physiological characteristics of H. segnis sp. nov., H. parasuis and H. paragallinarum*

All strains were negative in indole, urease, ornithine and lysine decarboxylases, arginine dihydrolase, β -glucuronidase and β -xylosidase. All were positive in the Porphyrin test.

	No. of positive strains			Negative strains*
	<i>H. segnis</i> (17 strains)	<i>H. parasuis</i> (9 strains)	<i>H. paragallinarum</i> (2 strains)	
H ₂ S (lead acetate)	0	5	0	All except HK417, 424, 425, 426 427 (weak reactions)
Haemolysis	0	0	0	—
Glucose, acid	w†	9	2	—
Glucose, gas	0	0	0	—
Maltose, acid	w	9	2	—
Sucrose, acid	w	9	2	—
Lactose, acid	0	0	1	HK381
Cellobiose, acid	0	0	1	HK381
Xylose, acid	0	0	2	—
Deoxyribose, acid	0	9	0	—
Inulin, acid	0	0	1	HK381
Mannitol, acid	0	0	2	—
β -Galactosidase	12	7	2	HK127, 129, 307, 316, 317, 422, 423
α -Glucosidase	0	4	2	HK362, 418, 422, 423, 425
β -Glucosidase	0	1	0	All except HK424
α -Fucosidase	0	9	1	HK381
Catalase	w	9	2	—
Oxidase	0	0	0	—
CO ₂ improves growth	0	0	0	—

* Only exceptions within individual species are listed.

† Weak to negative reactions (see text).

predominantly irregular filaments interspersed with a few bacillary forms. On chocolate agar the colonies were smooth or granular, highly convex, greyish-white, opaque and reached a diameter of about 0.5 mm. No odour was emitted from the growth. Satellite growth was obtained on both horse and calf blood agar around staphylococcal colonies or a NAD-containing disc. They were not haemolytic, and excess CO₂ was not required for good growth. The growth in carbohydrate media was sparse, and acid was not produced or produced in very small amounts (final pH 6.5 to 7.0; control tube without sugar: 7.2). Catalase and oxidase reactions were negative to weakly positive. The GC content of the DNA was the highest value obtained for any species of *Haemophilus*: 43.5 ± 0.54 mol % (Table 5; mean value for the four strains HK35, HK84, HK307 and HK316). The phenotypic characters are listed in Table 15.

Proposed type strain. Strain HK316 is proposed as type strain for *Haemophilus segnis* and has been deposited in the National Collection of Type Cultures as NCTC10977.

Haemophilus parasuis

Nine strains of porcine origin (Table 1) were tentatively labelled *H. parasuis* according to the proposal of Biberstein & White (1969). The problems of nomenclature for the group are discussed below.

Characteristics of H. parasuis. The properties differentiating *H. parasuis* from other V-factor-dependent species were its marked, but partly unknown, nutritional requirements,

the α -fucosidase activity, a positive catalase reaction, and a weak fermentation of carbohydrates (final pH 6.4 to 6.8) without production of gas from glucose. The following properties further characterized the strains assigned to this species: they were non-motile, predominantly thin, pleomorphic rods of varying length. Growth on chocolate agar was very feeble after 48 to 72 h incubation. The colonies were smooth, greyish, translucent and reached a diameter of about 0.5 mm. A marked enhancement of growth, with colonies attaining a diameter of 1 to 2 mm, was seen around contaminating organisms or a streak of a staphylococcus on the chocolate agar. The same growth stimulation on chocolate agar was not obtained around a NAD-containing disc. No haemolysis was seen on horse blood agar. Growth was not enhanced by excess CO₂ in the incubation atmosphere. The GC content of DNA of HK424 was 41.5 mol %. The phenotypic characters of the strains are listed in Table 15.

Haemophilus pleuropneumoniae

Fifteen strains of porcine origin were assigned to this species (Table 1). The serotypes of the strains quoted in Table 1 are as stated by the donor.

Characteristics of H. pleuropneumoniae. The strains assigned to *H. pleuropneumoniae* were V-factor-dependent and possessed the ability to synthesize porphyrins from δ -aminolaevulinic acid. The group was very homogeneous and corresponds to the organism designated *H. pleuropneumoniae* by Shope (1964) and *H. paraaemolyticus* by Nicolet (1968). The problems of nomenclature for the group are discussed below.

The production of urease and fermentation of mannitol, xylose and deoxyribose differentiated this species from other V-factor-dependent haemophili of porcine and human origin. The following properties characterized the group. All strains were non-motile coccobacilli or rather large rods, with only occasional filamentous forms. Two types of colonies, both opaque and reaching a diameter of 1.0 to 1.5 mm, were produced on chocolate agar. On primary isolation a rounded, hard, 'waxy' colony type predominated, which adhered to the platinum loop, but after several transfers on chocolate agar a flatter, soft, glistening colony was mainly found. All strains were capsulated and produced iridescent colonies on Levinthal's agar. None of the strains required CO₂ for good growth. They were all β -haemolytic on primary isolation, but five strains (HK355, 356, 358, 359 and 360) had lost the property on receipt. Carbohydrates were fermented to a final pH of 5.3 to 6.0. The GC content of their DNA (mean value for the three strains HK353, HK356 and HK358) was 42.0 ± 0.35 mol % (Table 5). The phenotypic characteristics of the strains are listed in Table 13 (under '*Haemophilus paraaemolyticus*: Porcine strains').

Strain HK361 has been deposited in the National Collection of Type Cultures as NCTC 10976.

Haemophilus paragallinarum

Haemophilus paragallinarum was represented by only two strains (Table 1), and they were only tentatively assigned to this species which was originally proposed by Biberstein & White (1969). The nomenclatural problems of the species are discussed below.

Characteristics of the strains. The two strains were in many ways similar to *H. parasuis*. The most useful features differentiating the two species were the stronger fermentative ability of *H. paragallinarum* (final pH 5.0 to 5.3) and the fermentation of several carbohydrates not metabolized by *H. parasuis* (Table 15). The two strains were short, non-motile rods with occasional filaments. Colonies on chocolate agar were smooth, greyish and translucent, and reached a diameter of 0.5 to 1.0 mm. The strains were not haemolytic and

Table 16. *Biochemical and physiological characteristics of three unnamed NAD-dependent taxa, A, B and C*

None of the strains produced β -glucosidase, β -glucuronidase, β -xylosidase or α -fucosidase. All strains were positive in the Porphyrin test.

	No. of positive strains			Negative strains*
	Taxon A (4 strains)	Taxon B (4 strains)	Taxon C (5 strains)	
Indole	4	4	0	—
Urease	0	4	0	—
Ornithine decarboxylase	4	4	0	—
Lysine decarboxylase	4	0	0	—
Arginine dihydrolase	0	0	0	—
H ₂ S (lead acetate)	4	4	5	Weak: HK164, 167, 382, 383, 384, 428, 429
Haemolysis	0	0	0	—
Glucose, acid	4	4	5	—
Glucose, gas	2	0	0	HK164, 170
Galactose, acid	NT	NT	5	—
Sucrose, acid	4	4	5	—
Lactose, acid	0	0	0	—
Xylose, acid	3	3	0	HK170, 447
Deoxyribose, acid	4	4	3	HK428, 429
Inulin, acid	NT	NT	3	HK383, 428
Mannitol, acid	0	0	0	—
Arabinose, acid	NT	NT	4	HK383
β -Galactosidase	0	4	0	—
α -Glucosidase	0	0	5	—
Catalase	4	4	5	—
Oxidase	0	4	0	—
CO ₂ improves growth	0	0	4	HK382
Capsule	3	0	0	HK170
Haemagglutination	3	0	0	HK167

NT, Not tested.

* Only exceptions within the individual taxa are listed.

did not require CO₂ for good growth. The GC content of DNA extracted from HK381 was 42.2 mol %. The biochemical and physiological features of the strains are summarized in Table 15.

Unnamed V-factor-dependent taxa

Among 13 V-factor-dependent strains not assigned to any of the taxa described above, three distinct groups were recognized. It is possible that these groups merit specific rank, but at present they will only be labelled taxon A, taxon B, and taxon C. Taxa A and B seem to be in an intermediate position between *H. influenzae* and *H. parainfluenzae*, possessing several characters of each of the two species.

Description of the individual taxa. Taxon A. The four strains in this taxon (Table 1) were short, non-motile rods with occasional filamentous forms. Colonies on chocolate agar were smooth, convex, greyish and translucent, and reached a diameter of 1 to 2 mm. The GC content of DNA from HK164 was 39.3 mol %. The characteristics of the strains are listed in Table 16.

Taxon B. These four strains (Table 1) were non-capsulated and non-motile coccobacilli and short rods, with occasional filamentous forms. Colonies on chocolate agar were smooth,

convex, white and opaque, and reached a diameter of 1.5 to 2.0 mm. The characters of the strains are summarized in Table 16.

Taxon C. The five strains in this taxon (Table 1) resembled *H. paraphrophilus* in many ways, for example in the requirement for CO₂, but differed by failing to ferment lactose and to produce gas from glucose broth. Some of the strains have been designated by others as *H. suis*, *H. parasuis* and *H. paragallinarum*, but they differed from other strains of these species by the ability to grow abundantly on chocolate agar, the appearance of the colonies, a distinct improvement of growth by CO₂, the failure to produce α -fucosidase and by the ability to ferment arabinose (Table 16). They were non-capsulated and non-motile, and were regular rods or occasionally filaments. Colonies on chocolate agar were granular, highly convex, yellowish and opaque, and reached a diameter of 1.0 to 1.5 mm. The GC content of their DNA (mean value for the two strains HK383 and HK428) was 42.0 mol % (Table 5).

Species rejected from Haemophilus: H. piscium

This species was represented by six strains (Table 1) originally isolated from trout ulcers and described by Snieszko, Griffin & Friddle (1950). The taxonomic position of the species is discussed below.

Characteristics of H. piscium. The six strains had identical characteristics but differed from all other strains of the collection in several ways. They were non-motile, small rods of varying length, with occasional filamentous forms. All strains were predominantly Gram-negative when stained, but with a few cells that resisted decolorization. This variation was especially marked in HK420. An examination of the fine structure of the cell walls of the strains revealed, however, that they were typical for a Gram-negative organism (Kilian & Theilade, 1975). The strains grew equally well on fish extract agar (Snieszko *et al.*, 1950) and on chocolate agar at 22 °C. No growth was obtained at 37 °C. On chocolate agar after 4 days incubation colonies were smooth, convex, whitish and translucent, and reached a diameter of 1 to 2 mm. They did not emit the characteristic sweetish smell of most haemophili. The main characters of the strains are summarized in Table 4. Characters not included in the Table are: arginine dihydrolase negative, negative in glycosidases except for a weak positive reaction of β -galactosidase after 24 h, carbohydrates fermented to a final pH of 5.2. Nitrate was not reduced, contrary to the information given in *Bergey's Manual of Determinative Bacteriology* (1957) but in agreement with the findings of Snieszko *et al.* (1950). The GC content of their DNA (mean value calculated for the two strains HK420 and HK421) was 55.1 mol % (Table 5).

Strains of related genera

A list of strains of the species *A. actinomycetemcomitans*, *Ei. corrodens* and *P. multocida* included in the study for comparative purposes is given in Table 1. These three species all consist of non-motile, Gram-negative pleomorphic rods which resemble especially *H. aphrophilus* and *H. haemoglobinophilus*, and therefore problems in differential diagnosis may arise. The most useful differentiating features between these species are summarized in Table 17. All the strains of *Ei. corrodens* were shown to be identical with regard to the properties tested as were the strains of *P. multocida*. These results are shown in Table 17. The strains of *A. actinomycetemcomitans* are described in detail in the following paragraph, with special reference to the relationship to *H. aphrophilus*.

Actinobacillus actinomycetemcomitans. All strains examined were small coccobacilli with only occasional filamentous forms. Colonies on chocolate agar were usually smaller and

Table 17. *Characters of diagnostic value for the differentiation of H. paraphrophilus, H. aphrophilus, H. haemoglobinophilus, and selected species of related genera*

	No. of strains	ALA → porphyrins	NAD-requirement	Indole	Urease	Ornithine decarboxylase	Lysine decarboxylase	Glucose, acid	Glucose, gas	Sucrose, acid	Lactose, acid	Mannitol, acid	Xylose, acid	β-Galactosidase (ONPG)	α-Glucosidase (PNPG)	Alkaline phosphatase	Catalase	CO ₂ improves growth
<i>H. paraphrophilus</i>	11	+	+	—	—	—	—	+	+	+	+	—	—	+	—	+	—	—
<i>H. aphrophilus</i>	16	+	—	—	—	—	—	+	+	+	+	—	—	—	—	+	—	—
<i>H. haemoglobinophilus</i>	5	—	—	+	—	—	—	+	—	+	—	+	+	—	—	+	+	—
<i>A. actinomycetem-comitans</i>	7	+	—	—	—	—	—	+	—	—	—	+	—	—	—	+	+	+
<i>Ei. corrodens</i>	5	+	—	—	—	+	+	—	—	—	—	—	—	—	—	—	+	+
<i>P. multocida</i>	3	+	—	+	—	+	—	+	—	+	—	+	+	—	+	+	+	—

For symbols, see Table 4.

Table 18. *Correlation between origin and species assignment of 367 Haemophilus strains isolated from humans*

The figures in parentheses indicate the number of strains possessing a capsule.

	Total no. of strains	<i>H. influenzae</i> , biotype I	<i>H. influenzae</i> , biotype II	<i>H. influenzae</i> , biotype III	<i>H. influenzae</i> , biotype IV	<i>H. influenzae</i> , biotype V	<i>H. haemolyticus</i>	<i>H. parainfluenzae</i>	<i>H. paraphrophilus</i>	<i>H. aphrophilus</i>	<i>H. segnis</i>	Unnamed taxon A
Upper respiratory tract	81	9 (2)	20	8	5 (1)	—	8	30	—	—	—	—
Oral cavity	105	2	1	—	—	—	—	72	8	6	16	—
Meningitis	47	39 (39)	5 (4)	1	2 (2)	—	—	—	—	—	—	—
Blood cultures and epiglottitis	6	6 (6)	—	—	—	—	—	—	—	—	—	—
Otitis media	29	9 (2)	9	1	—	10	—	—	—	—	—	—
Conjunctivitis	43	1 (1)	16 (1)	17	—	—	—	5	—	—	—	4
Respiratory infections	21	4 (2)	10 (1)	—	—	—	1	4	1	1	—	—
Endocarditis	6	—	—	—	—	—	—	—	—	6	—	—
Pus from abscesses and wound infections	10	—	—	—	—	—	—	5	2	3	—	—
Other infections, or origin unknown	11	1	3 (2)	1	5 (4)	—	—	1	—	—	—	—
Vaginal and urethral cultures	8	—	—	—	—	—	—	8	—	—	—	—

more adherent, but otherwise resembled those of *H. aphrophilus*. Excess CO₂ in the incubation atmosphere was essential for growth on chocolate agar; *H. aphrophilus* was less demanding in this respect. None of the strains was haemolytic. Growth in broth was granular and adhered to the tube as described for *H. aphrophilus*. The GC content of DNA extracted from HK435 was 42.7 mol %. The phenotypic characters of the strains are listed in Table 14.

Observations on the correlation between source and species

For the 367 isolates of human origin, Table 18 shows the correlation between the origin and the specific groups to which the *Haemophilus* strains were assigned. The clinical diagnoses summarized in this Table are mainly those provided by the donor of each strain. Verification through the original case sheets of the patients has not been attempted, since for most this would be impossible. The strains from oral cavities and conjunctivae were selected from a larger number of strains, so that the collection included in this study would be of a suitable composition; therefore, the relative composition of these parts of the collection does not reflect the composition of the original materials. A proportion of the strains from conjunctivae were not epidemiologically independent and some of the strains may have originated from a single strain. With these reservations in mind, Table 18 demonstrates that all strains isolated from meningitis, blood cultures, epiglottitis and otitis media belonged to *H. influenzae*, whereas almost all strains isolated from oral cavities and from vaginal and urethral cultures belonged to the haemin-independent species. This latter group of species also encompassed most strains cultured from pus from abscesses and wound infections. Many of these infections were directly or indirectly related to the oral cavity (Table 1).

DISCUSSION

Early studies on the haemophilic bacteria revealed a number of important characteristics which they had in common, as well as characteristics which differentiated between them, such as differences in serology, haemolytic ability and various biochemical properties (Jordan, 1919; Pritchett & Stillman, 1919; Yabe, 1921; Kristensen, 1922). After recognition of the two distinct growth factors, generally known as X and V (Davis, 1917; Fildes, 1921; Thjøtta & Avery, 1921), and the differences in the requirement for them (Rivers, 1922*a, b*; Valentine & Rivers, 1927), one of the common features of all attempts to classify the *Haemophilus* group has been the overriding weight placed on these requirements. Only a few other properties, e.g. the haemolytic ability, have been accepted as useful in the subdivision of the genus.

Growth factor requirements

The V-factor is closely concerned in oxidation-reduction processes of the growing cell (Pittman, 1935) and is one of the two co-dehydrogenases, NAD or NADP (Lwoff & Lwoff, 1936*a, b*, 1937*a, b*) or certain undefined precursors to these (Gingrich & Schlenk, 1944; Shifrine & Biberstein, 1960). The value of differentiating haemophili on the basis of this requirement is generally accepted and its determination does not usually give rise to problems. The satellite phenomenon originally described by Grassberger (1897) provides a simple way of determining the V-factor requirement, but the method should not be used uncritically since other factors produced by the feeder organism can be responsible for a satellite phenomenon (Ryan, 1968) as confirmed here. This problem could probably be avoided by the application of NAD-containing discs in place of a staphylococcus, although the possibility exists that occasional strains are unable to take up the NAD molecule. The V-factor excreted by feeder bacteria, where it has been examined, has turned out to be some precursor to NAD (Shifrine & Biberstein, 1960). To obtain a valid result, a basal medium free of available V-factor must be used. Blood agar contains variable amounts of V-factor, depending on the freshness of the medium and on uncontrollable, individual factors related to the medium. The unsatisfactory satellite phenomenon often seen in this study, especially on horse blood

agar, confirms this. Thus it is possible that the use of blood-containing media for the primary screening of strains may result in an erroneous rejection of some strains as being independent of V-factor.

The haemin requirement of some *Haemophilus* strains has been the subject of extensive studies which have contributed to the general understanding of the respiratory systems in micro-organisms. Lwoff & Lwoff (1937*c*) showed that haemin is used by *H. influenzae* in the biosynthesis of the iron-containing respiratory enzymes, cytochrome, cytochrome oxidase, catalase and peroxidase. Haemin is apparently required for growth under any conditions, but much less is required under anaerobic incubation (Gilder & Granick, 1947). Various precursors in the haemin biosynthetic pathway such as protoporphyrin IX or other iron-free porphyrins can normally be substituted for haemin, provided they contain vinyl side-chains (Gilder & Granick, 1947; Brumfitt, 1959). It is evident, however, that some of the iron-free precursors of haemin are utilized with relative difficulty and reduce some of the functional activities of the bacterial cells which involve haem enzymes such as catalase and nitrate reductase (Smith, Hale & O'Callaghan, 1953). Some strains of *Haemophilus* do not have the enzymic capacity to incorporate iron in the protoporphyrin molecule and thus have a requirement for haemin as such (White & Granick, 1963; Kilian *et al.*, 1976). However, like most other micro-organisms, strains of the species *H. parainfluenzae*, *H. aphrophilus*, *H. piscium* and *H. suis* can form the cytochrome system without a supply of haemin (White & Granick, 1963).

Although generally accepted, the taxonomic significance of haemin requirement has never clearly been established, mainly because of the paucity of other independent characteristics by means of which a correlation can be demonstrated. The value of differentiating haemophili on the basis of differences in this requirement has in fact been questioned by some. Smith *et al.* (1953) considered a sharp division of haemophili into haemin requiring and non-haemin requiring ones an oversimplification, and suggested that *H. parainfluenzae* may merely represent one end of a wide series of *H. influenzae* strains which range from those completely incapable of haemin synthesis to those which can synthesize sufficient haemin for all their requirements, even under aerobic growth. Their theory was based on the findings that strains of *H. influenzae* require different amounts of X-factor when grown anaerobically in infusion broth, and that some strains contain traces of the haem enzymes nitrate reductase and catalase even when grown in the absence of X-factor under these conditions. The ability of some strains to grow in a broth medium without haemin, however, suggests the presence of minute amounts of X-factor in the basal medium used by Smith *et al.* (1953) or transferred with the initial inoculum into the medium. Therefore the traces of the haem enzymes found do not necessarily indicate that bacteria are able to synthesize haemin. Studies on catalase activity of *Haemophilus* strains, however, led Biberstein & Gills (1961) to adopt the theory of Smith *et al.* (1953). Catalase production is a variable property in haemophili, as the present study has confirmed, and it is in varying degrees dependent on the presence of haemin, even in haemin-independent haemophili. Furthermore, it is highly dependent on the growth phase and growth condition of the bacteria (White & Smith, 1962; White, 1962). Biberstein & Spencer (1962) suggested a rather subordinate role for catalase in the terminal oxidative processes of *Haemophilus* strains. The activity of catalase, although a haem enzyme, is therefore a poor marker for the haemin requirement of haemophili. Reports on haemin-independent mutants of haemophili usually considered to be haemin-dependent (Butler, 1962; Boyce, Frazer & Zinnemann, 1968) have questioned its differential value. Comparative studies of the formation of porphyrins and of the regulation of the electron-transport systems seem, however, to indicate basic differences between haemin-dependent

and -independent haemophili (White & Granick, 1963; Biberstein, Mini & Gills, 1963; White, 1962, 1963). The difference between *H. influenzae* and *H. parainfluenzae* has been stressed by recent genetic studies of strains of the two species. Beattie & Setlow (1970) found a low ratio of hetero- to homospecific transformation frequency in the two species, and Boling (1972) only found a 44 % homology between DNAs from a strain of *H. influenzae* and *H. parainfluenzae* as revealed from DNA-DNA hybridization experiments. The quantitative differences in the haemin requirement experienced by many might be explained by the findings by Gilder & Granick (1947). Small inocula of *H. parainfluenzae* often do not grow in culture media unless supplied with haemin, whereas large inocula grow readily in media devoid of added haemin. This is probably due to a constant inactivation of haemin by traces of peroxides produced in the culture medium during growth. The ability of haemin-independent haemophili to grow in media devoid of haemin is therefore not only dependent on the rate at which the bacteria synthesize haemin but also on the rate of its inactivation.

The heavy weighting normally placed on haemin requirement as a tool in the identification and classification of haemophili is in contrast to the difficulties experienced in an exact determination of the property (Gilder & Granick, 1947; Kilian *et al.*, 1972). This has been the main problem in *Haemophilus* taxonomy and undoubtedly accounts for much of the equivocal information on the biology of the species. Instead of the usual determination of haemin requirement by growth experiments, the present study has tested the ability of all strains to synthesize porphyrins from δ -aminolaevulinic acid (Biberstein *et al.*, 1963; Kilian, 1974). A primary subdivision of strains on the basis of this character showed a perfect correlation with DNA base composition, fermentation reactions and several other independent biochemical properties. This fact, together with the reliability of the porphyrin test, makes this property the most suitable character in the primary subdivision of the genus *Haemophilus*.

Requirement for carbon dioxide

The CO₂ requirement has been used as one of the primary characters in establishing the species *H. aphrophilus* (Khairat, 1940), *H. paraphrophilus* (Zinnemann *et al.*, 1968), and *H. paraphrohaemolyticus* (Zinnemann *et al.*, 1971). In this study, a raised CO₂ tension in the incubation atmosphere was obtained in an anaerobic jar. It should be stressed that this method does not exclude possible growth stimulation due solely to the increased humidity obtained in a jar. Nearly all strains assigned to *H. aphrophilus* and *H. paraphrophilus*, in addition to strains of taxon C, grew better in air supplemented with 10 % CO₂. The same improvement of growth was also noted with a few strains assigned to other groups, but such strains were otherwise similar to the other strains of the taxonomic groups to which they were assigned. *Haemophilus paraphrohaemolyticus* has thus not been listed as a separate species. The study indicates that a CO₂ requirement is a valuable criterion for the identification of some species of *Haemophilus*, when allied to other characters.

Haemolysis

This property has been used as a primary character for the recognition of the species *H. haemolyticus* and *H. parahaemolyticus* (Pittman, 1953). Several of the strains included in this study, haemolytic on primary isolation, lost the property after one or more subcultures, with consequent problems in the classification of some of the NAD-dependent strains. Whereas haemolytic strains requiring both haemin and NAD differed in several ways from strains of *H. influenzae* (Table 4), NAD-dependent haemolytic strains of human origin which had lost their haemolytic ability were indistinguishable from the corresponding

non-haemolytic strains (Table 13). The instability of haemolysis was also noted with strains assigned to the species *H. pleuropneumoniae*, but this species was fairly well defined by many other phenotypic characters (Table 13). These findings suggest that haemolysis is an unsatisfactory criterion for establishing a species. Consequently, the property was not used for the primary subdivision of NAD-dependent strains of human origin, and strains showing haemolysis were included in biotypes II or III of *H. parainfluenzae* established on other characters (Table 12).

Fermentation of carbohydrates

The value of fermentation reactions in the differentiation of *Haemophilus* species revealed by the present study was surprising as it conflicts with generally held opinion. Most species were relatively strong acid producers. Clear-cut and reproducible results were obtained with the commercial fermentation medium used. Kristensen (1922), McGaughey (1932) and Tunevall (1951) have noted the ability of haemophili to produce acid in media devoid of sugar. Although confirmed by the present study, the increase in acidity of non-glycolytic origin never reached quantities sufficient to disturb the readings. However, fermentation experiments with haemophili, at least to some degree, seem to be dependent on the medium used. This is evident from a comparison of some of the results obtained here with those of Johnson (1972), for strains common to the two studies.

The production of gas in glucose media by strains of some species is also important. Except for some conflicting reports on *H. aphrophilus* (King & Tatum, 1962; Boyce *et al.*, 1968; Sutter & Finegold, 1970), most authors describe haemophili as fermenting carbohydrates with the production of acid but no gas. The results of the present study are, however, a confirmation of the early and obviously overlooked findings of Stillman & Bourn (1920). Although they were ignorant of the differences in growth requirements, their classification, based on gas production and fermentation of sucrose along with indole production and haemolysis, is in many ways similar to the one obtained in the present study. Most strains of *H. haemolyticus*, *H. parainfluenzae*, *H. paraphrophilus* and *H. aphrophilus* produced relatively small but definite amounts of a mixture of hydrogen and carbon dioxide from glucose, indicative of a mixed acid fermentation. The fact that the remaining species were anaerogenic indicates differences in the pathways of carbohydrate metabolism within the genus *Haemophilus*. The lack of gas production by strains of biotype III of *H. parainfluenzae*, *H. segnis* and *H. parasuis* were, however, associated with a less pronounced production of acid. Hydrogenlyase is induced only at a pH of 6 or less (Blackwood, Neish & Ledingham, 1956), and the findings for these taxa therefore do not exclude a potential hydrogenlyase activity. Consequently, the taxonomic significance of the latter findings cannot be fully evaluated until further knowledge of the carbohydrate metabolism of these and other *Haemophilus* species is obtained.

The gas produced by most aerogenic strains in nitrate broth also consisted of hydrogen and carbon dioxide, and should not be taken to indicate a denitrification activity. The significantly reduced amounts evolved at higher nitrate concentrations can be explained by the inhibitory effect of nitrate on the hydrogenlyase activity (Billen, 1951; Pinsky & Stokes, 1952).

Taxonomy and nomenclature

General comments. Ideally, the taxonomy of a bacterial group is based on an unbiased analysis of a large set of properties of many strains of different origin. None of these ideal requirements has been totally met in the present study. As regards strains of human origin,

the collection studied was probably sufficiently large and as representative as desirable. Unfortunately this is not true for the strains isolated from animals: haemophili can be isolated from many animal species not considered in this study.

The fairly large number of biochemical tests used in the study, of which several have not previously been applied to haemophili, revealed a number of hitherto unrecognized characters of taxonomic significance and have increased the information on the general properties of the genus. Considering the heterogeneity of the strain material, the number of properties determined was close to the minimum necessary for the recognition of so many different species, but even the use of this limited set of easily performed tests established a clear-cut distinction between most of the individual species (Table 4). Several of these can now be more accurately defined, but further characterization of some of the species is still desirable, particularly for the possible subdivisions or biotypes of *H. influenzae* and *H. parainfluenzae*.

The recent numerical taxonomic study by Sneath & Johnson (1973) included the determination of a large number of characters. Although the usefulness of some of these characters may be debatable, the main limitation to their study was the small number of strains included, particularly because it can now be seen that several of the *Haemophilus* species are more heterogeneous than hitherto recognized. The results obtained in the present study would make it possible to select a relatively small but representative collection of strains for more extensive studies.

Although type strains are available for only a few of the *Haemophilus* species and often the description of the species has been inadequate, it has usually been possible to equate the preliminary groups with previously described named species. However, some of them pose problems that will now be discussed separately for the individual species.

Haemophilus influenzae. A type or neotype strain of the type species *H. influenzae* (Lehmann & Neumann, 1896) Winslow *et al.*, 1917 has unfortunately never been designated. Sneath & Johnson (1973) state that the organism given the name is well known, and that numerous strains could be accepted for that purpose. The results obtained for *H. influenzae*, however, demonstrate the heterogeneity of this species, which has been repeatedly suggested by strain-specific differences in pathogenicity (Rosher, 1931), various biochemical properties such as indole formation (Jordan, 1919; Rhein, 1919; Rivers, 1920; Yabe, 1921), fermentation of carbohydrates (Stillman & Bourn, 1920; Smith, 1931; *Bergey's Manual of Determinative Bacteriology*, 1957), urease and lysine decarboxylase activity (Huet, 1959) and antigenic constitution (Pittman, 1931; Wilson & Miles, 1964; Turk & May, 1967). Rivers & Kohn (1921) and Kristensen (1922) noted that indole production is more often encountered in meningeal than in respiratory strains of *H. influenzae*, and Rosher (1931) showed that the property is correlated to some extent with pathogenicity under experimental conditions. Together with a few other biochemical properties, indole production was used here in the differentiation of 188 *H. influenzae* strains into five biotypes (Table 4). The value of distinguishing between these biotypes is indicated by the positive correlation with the sources of the strains. Eighty-five per cent of the strains isolated from meningitis and blood cultures, including cases of epiglottitis, could be assigned to biotype I, whereas all except one of the strains from conjunctivitis were assigned to biotypes II and III, which also contained most of the respiratory strains (Table 18). The distinguishing characters of the five biotypes are few, and a more extensive phenotypic characterization is desirable.

One of the important characters of haemophili causing meningitis and other acute infections is the production of a capsule. Capsule formation can be reintroduced in converted rough *H. influenzae* strains by transformation (Alexander & Leidy, 1950, 1951*a, b*). Only a few respiratory strains of *H. influenzae* can, however, be transformed in this way (Tunevall,

1952) in spite of the fact that such strains are often transformable to streptomycin resistance. It is possible that these findings are reflexions of the heterogeneity of *H. influenzae*, which might explain the different incidence of capsulation found in the five biotypes (Table 8). Studies of frequency of transformation of genetic determinants of capsule production and antibiotic resistance between the biotypes may throw some light on this problem and provide more information on the relationship between the biotypes.

Some of the strains assigned to biotypes II and III of *H. influenzae* were isolated from eye swabs from Tunisian, Egyptian and Danish patients with conjunctivitis. As pointed out by Huet (1956), it has been the custom to label all haemophili recovered from eyes as Koch-Weeks bacilli or *H. aegyptius*, though plain *H. influenzae* strains can be isolated from eye infections as well (Pittman & Davis, 1950; Rogers, Zinnemann & Forster, 1960). The problems of distinguishing the Koch-Weeks bacillus from *H. influenzae* have been discussed in earlier papers (Pittman & Davis, 1950; Ingham & Turk, 1969). Pittman and co-workers apparently had clarified the problems when reporting on a number of differences in serology, in biochemical reactions, in growth characteristics, and in the ability to agglutinate red blood cells. They showed that *H. aegyptius* is serologically more homogeneous than non-capsulated *H. influenzae*, that it never forms indole or ferments xylose (properties which may also be encountered in strains of *H. influenzae*) and that it is haemagglutinating (Pittman & Davis, 1950; Davis *et al.*, 1950). It has been stated that the cellular morphology alone is sufficient for differentiation; however, Pittman & Davis (1950) found overlapping in size, although on the whole *H. aegyptius* tended to form more slender rods.

The validity of haemagglutination as a distinguishing character has been questioned by Ivler, Preston & Portnoy (1963) on the grounds that even *H. influenzae* can cause haemagglutination if the procedure is suitably modified. The present study shows that haemagglutination is a character almost exclusively encountered in conjunctivitis strains. Yet it is also seen in occasional strains of *H. parainfluenzae* (Sims, 1970) and apparently in *H. suis* (Bakos, 1955), although the latter finding was not confirmed with our procedure. The haemagglutinating ability in conjunctivitis strains was, however, not restricted to non-xylose fermenting strains, and the delineation between *H. aegyptius* and *H. influenzae* is thus further blurred. The close relationship between these two organisms is also indicated by transformation experiments (Leidy, Hahn & Alexander, 1959; Leidy, Jaffee & Alexander, 1965).

Specific recognition of Koch-Weeks bacillus is therefore probably not justified. Strains received as *H. aegyptius* and other strains resembling them were included in biotype III of *H. influenzae*, together with the suggested working type of *H. influenzae* (Sneath & Skerman, 1966). In agreement with these findings *Bergey's Manual of Determinative Bacteriology* (1974) lists *H. aegyptius* under *species incertae sedis*.

The so-called 'atypical *H. influenzae*' (Smith, 1931; Wilson & Miles, 1964), showing predominantly bacillary or filamentous morphology and fermenting sucrose, probably represents erroneously classified strains belonging to the *H. parainfluenzae* group. *Bergey's Manual of Determinative Bacteriology* (1974) lists sucrose fermentation as one of the characteristics of *H. influenzae*, but the present study has shown that, with the exception of *H. haemoglobinophilus*, sucrose fermentation is an exclusive character of haemin-independent species.

Haemophilus haemolyticus. The species *H. haemolyticus* (Bergey *et al.*, 1923) was based on the beta-haemolytic 'Bacillus X' of Pritchett & Stillman (1919). The growth requirements of the organism were not determined by these authors, but an interpretation of the biochemical reactions of 29 strains studied by Stillman & Bourn (1920) indicates that the name was used for both haemin-dependent and haemin-independent haemolytic strains. More

recently Pittman (1953) proposed that the name should be used for haemin- and NAD-dependent strains only.

The present study has shown that, in contrast to the haemin-independent haemolytic haemophili of human origin, *H. haemolyticus* forms a valid species well separated from other specific groups.

Strain HK385 was suggested as the working type for the species by Sneath & Skerman (1966), but has recently been found to be independent of haemin (Biberstein & Zinnemann, 1971; Kilian, 1974). In another culture (ATCC10014) of the same strain, White & Granick (1963) found all the enzymic activities characteristic of the haemin-independent species but were unable to grow the strain on a simplified medium free of haemin. This finding probably explains why Sneath & Johnson (1973) describe the strain as haemin-requiring, and it exemplifies the disadvantages of determining this requirement by growth experiments. In Sneath & Johnson's (1973) study the strain fell within the same cluster as *H. influenzae* but joined the cluster at a rather low level. However, the strain has the typical fermentation reactions of the *H. parainfluenzae* group, and the relatively high GC content of its DNA confirms the affiliation to that group. Here the strain was assigned to biotype III of *H. parainfluenzae*.

Haemophilus haemoglobinophilus (*canis*). This species is fairly well circumscribed by several independent phenotypic traits. It is also readily separated from *H. influenzae*, although Sneath & Johnson's (1973) study revealed a high degree of similarity between the two species.

The organism was originally isolated by Friedberger (1903) from dogs suffering from a suppurative inflammation of the prepuce, but it is usually regarded as a harmless parasite of the preputial sac because it can also be isolated from normal dogs (Kristensen, 1922; Rivers, 1922*b*). Until recently it has been isolated only from dogs, but Frazer & Rogers (1972) isolated a strain from otitis media in an agammaglobulinaemic child with no known contact with dogs.

Friedberger (1903) proposed the name *B. haemoglobinophilus canis* for the organism but it is frequently referred to as *H. canis* as proposed by Rivers (1922*b*). However, *Index Bergeyana* (1966) lists the correct name as *Haemophilus haemoglobinophilus* (Lehmann & Neumann, 1907) Murray, 1939, *H. canis* being a later synonym.

One of the strains included in this study (NCTC1659 = ATCC19416) has been stated to be a cotype of the species (Sneath & Skerman, 1966). Whereas the NCTC culture was typical of the species, the ATCC culture differed in one important way, namely in being consistently indole negative. It was identical to NCTC1659 in all other ways, and is likely to be a mutant of the original strain of Kristensen (1922).

Haemophilus ducreyi. Many reports on this organism have stressed the difficulties experienced in growing it. It is therefore not surprising that the information on its other properties is sparse and contradictory, and that it has been customary to rely on cultural and microscopic characteristics when identifying strains isolated from chancroid. About the action of *H. ducreyi* on carbohydrates there are conflicting reports. Whereas Reymann (1949*b*) and Ajello *et al.* (1956) did not find any fermentative ability, Hababou-Sala (1925) observed fermentation of glucose, sucrose and maltose, and de Assis (1926) noted fermentation of glucose. *Haemophilus ducreyi* is generally considered to be haemophilic, but the experimental results are equivocal and there are still doubts about the growth requirements of the organism. Several authors have reported that a thermostable serum factor and a thermolabile factor bound to erythrocytes is needed, whereas neither haemin nor yeast extract (NAD) have any effect on growth (Sanderson, 1940; Beeson, 1946; Reymann, 1951; Ajello

et al., 1956). In contrast, Lwoff & Pirotsky (1937) showed that the addition of haemin to a proteose peptone medium secured growth of their strains and they concluded that *H. ducreyi* resembles *H. haemoglobinophilus* in requiring haemin. Even the available information on the Gram-reaction is ambiguous. Nicolau & Banciu (1926) reported that after a number of subcultures their strains became Gram-positive although they were markedly Gram-negative on primary isolation. de Assis (1926) found that the Gram-reaction was dependent on the method used for fixing of the preparation before the staining procedure and Reymann (1951) found all his newly isolated strains consistently Gram-negative. This obvious discrepancy seemed to be clarified when Deacon *et al.* (1954) reported on a Gram-positive and non-pathogenic phase of the Ducrey bacillus, but disappointingly they later realized that they had been working with strains of *Corynebacterium acnes* (Deacon *et al.*, 1956). All these different findings reflect the uncertainty about the true nature of *H. ducreyi*. It is likely that the name has been used for several different bacteria. The present study partly confirms this assumption. None of the Reymann (1947, 1949*a*, *b*, 1950, 1951) strains studied here can be assigned to the genus *Haemophilus* as they have been shown to be Gram-positive (Kilian & Theilade, 1975). However, the two strains from the Collection de l'Institut Pasteur conform to the present definition of the genus *Haemophilus*, and the DNA base composition of these strains falls within the limits accepted for *Haemophilus* strains. The taxonomic position of these strains within the genus *Haemophilus* is therefore fully justified. However, it is an open question which of these two groups is the Ducrey bacillus and the causative agent of chancroid. Only the CIP strains showed the characteristic streptobacillary morphology usually ascribed to *H. ducreyi* (Unna, 1892) but Reymann (1951) was able to produce typical chancroids by inoculation of some of his strains in two volunteers. If the specific name *H. ducreyi* (Neveu-Lemaire, 1921) Bergey *et al.*, 1923 is to be conserved, it would be logical to use it for the Gram-negative haemin-dependent organism.

Haemophilus parainfluenzae. The specific name *Haemophilus parainfluenzae* was proposed by Rivers (1922*a*) for 'influenza-like bacilli which need only the heat-labile accessory food factor as present in unautoclaved yeast extract'. Fleming & Maclean (1930) and Sims (1970) isolated *H. parainfluenzae* from the oral cavities of all subjects studied, but except for a few well-documented cases of endocarditis caused by *H. parainfluenzae* (Miles & Gray, 1938), this organism has remained of little clinical significance. Turk & May (1967) believed that 'in general there is little point in attempting to distinguish between non-capsulated strains of the two species (*H. influenzae* and *H. parainfluenzae*), especially as haemophili totally independent of X-factor are undoubtedly rare and are, so far as we know, no different from *H. influenzae* in their clinical significance'.

This study has shown that *H. parainfluenzae* is a valid species well differentiated from *H. influenzae* by several phenotypic characters. Furthermore, the results indicate that the pathogenicity and habitat of the organism is significantly different from that of *H. influenzae*.

As Sims (1970) suggested, *H. parainfluenzae* is a heterogeneous group. Here the species was tentatively subdivided into three biotypes differentiated by a few biochemical features. The inclusion of biotype III in *H. parainfluenzae* is still under consideration. It differs from the other two biotypes in its failure to produce gas in glucose broth, and by a less pronounced acid production from the carbohydrates attacked. If future studies show that this is indicative of significant differences in the pathway of carbohydrate metabolism, biotype III would probably merit specific rank.

Valentine & Rivers (1927), in an attempt to classify the haemophilic bacteria, proposed that all haemophili that require both X and V be classified as *B. influenzae* (*H. influenzae*), whether haemolytic or non-haemolytic, and that those that require V factor only be classified

as *B. parainfluenzae* (*H. parainfluenzae*), whether haemolytic or non-haemolytic. This recommendation was adopted by Miles & Gray (1938) and Wilson & Miles (1964), and partially by Sims (1970). However, Pittman (1953) stated that apart from the growth requirements, 'there is no evidence to indicate that haemolytic bacteria should be classified as a variety of the non-haemolytic bacteria', and she accordingly proposed to separate the haemolytic haemin-independent strains from *H. parainfluenzae* and thus to subdivide haemolytic haemophili into *H. haemolyticus* and *H. parahaemolyticus*.

The present study shows that, whereas the species *H. haemolyticus* is fairly distinct from *H. influenzae*, the specific assignment of haemolytic NAD-dependent haemophili of human origin is highly questionable. The only distinguishing character of such strains, their haemolytic ability, seems to be unstable. The proposal of Valentine & Rivers (1927) concerning the NAD-dependent haemophili has therefore been adopted, and haemolytic strains of this kind have been included in biotypes II and III of *H. parainfluenzae*.

Thirty-nine per cent of the strains assigned to biotype III required CO₂ for good growth. The strains included those of Zinnemann *et al.* (1971) who designated one of them as the holotype of *H. paraphrohaemolyticus*. The growth-stimulating effect of CO₂, however, was not restricted to haemolytic strains. Therefore in my opinion *H. paraphrohaemolyticus* as described at present is not a valid species, but when more information on the carbohydrate metabolism of haemophili is obtained it is possible that biotype III as a whole will merit specific status.

One of the strains (HK416) assigned to biotype I was received as a cotype of *H. paraphrophilus*. This strain shared all the characteristics of biotype I of *H. parainfluenzae* and had none of the distinguishing characteristics of *H. paraphrophilus*. Furthermore, the GC content of the DNA of the strain was significantly lower than that of *H. paraphrophilus*. Another culture of the same strain had identical characteristics and it should consequently be rejected as a strain of *H. paraphrophilus*.

One of the strains assigned to biotype II of *H. parainfluenzae* deserves special attention. The ICNB Subcommittee on the Taxonomy of *Haemophilus* regarded Pittman's strain 734 (HK339) as a neotype strain of *H. haemolyticus* (Zinnemann, 1971), in place of the suggested working type strain NCTC8479 (HK385) (Sneath & Skerman, 1966) which was rejected when it was realized that it is haemin-independent (see under *H. parainfluenzae* biotype III). In fact, another strain was subsequently placed in the National Collection of Type Cultures (NCTC10659) by Zinnemann as a true *H. haemolyticus* because, on detailed investigation, Pittman's strain 734 was found not to be X-dependent but only V-dependent (K. Zinnemann, personal communication). This is confirmed by the present study.

Haemophilus aphrophilus and *H. paraphrophilus*. The two CO₂-requiring species *H. aphrophilus* and *H. paraphrophilus* are similar in nearly all respects, but whereas *H. paraphrophilus* meets the criteria for the inclusion in the genus *Haemophilus* as defined by the Subcommittee on the Taxonomy of *Haemophilus* (Zinnemann, 1967), *H. aphrophilus* does not meet these criteria as it is capable of synthesizing both NAD and haemin. Khairat (1940) reported dependence on haemin for growth on primary isolation, but with a gradual reduction of the stringency of the requirement with prolonged subculture. Several authors have obtained similar results, and all agree that there is a fairly high rate of mutation to haemin-independence, although there are strain variations (Toshach & Bain, 1958; Boyce *et al.*, 1968; Sneath & Johnson, 1973). King & Tatum (1962) found that the haemin requirement 'is certainly not an outstanding characteristic of this organism', and Sutter & Finegold (1970) reported that their isolates of the species were independent of haemin. This view is supported by the results of White & Granick (1963), who showed that one of Khairat's

(1940) strains had all the enzymes of the haemin biosynthetic pathway characteristic for haemin-independent species. All strains of *H. aphrophilus* included in the present study also possessed the enzymic capacity to convert δ -aminolaevulinic acid to porphyrins. Nearly all these strains were stock strains, but because of the many enzymic steps involved in this biosynthetic pathway, it is highly unlikely that the porphyrins, which can be detected after incubation, can be explained as having been synthesized by mutants. The reason for an apparent need for haemin-containing media at primary isolation remains to be elucidated.

The taxonomic position of *A. actinomycetemcomitans* is uncertain. The species does not fit the criteria for the genus *Actinobacillus* in *Bergey's Manual of Determinative Bacteriology* (1974), and is referred to as a *species incertae sedis*. It shares many of the characters of the aphrophilus group (Table 14), and seems to be more closely related to *H. aphrophilus* than to *A. lignieresii*, the type species of the genus *Actinobacillus* (King & Tatum, 1962). Considering this fact, and the similarity between *H. aphrophilus* and *H. paraphrophilus* (a 'true *Haemophilus*'), it is possible that all three species should be in the genus *Haemophilus*, although this would require a change in the definition of the genus. Sneath & Johnson (1973) showed that various strains of *Actinobacillus* were more closely related to *Pasteurella* strains than to strains of *Haemophilus*, but their study unfortunately did not include strains of *A. actinomycetemcomitans*. At present, therefore, it is not possible to draw any definite conclusions as to the systematic position, and studies of nucleic acid homology are probably required to solve the question.

The three species usually require the addition of CO₂ to the environment for growth, though the requirement is not invariable as in other CO₂-requiring bacteria, for example *Brucella abortus* (Bouvier, 1960). Thus stock strains of *H. aphrophilus* frequently become adapted to growth in the absence of CO₂ (Khairat, 1940) and some of the strains assigned to the species *H. aphrophilus* and *H. paraphrophilus* in this study did not show the requirement. Accordingly, the property should not be used as a primary diagnostic character in the identification of strains of these species.

Haemophilus segnis sp. nov. The relatively high %GC (43.5) is one of the main characteristics of the NAD-dependent strains for which the name *H. segnis* is proposed. Several other characters, mainly of a negative nature, distinguish this species from other NAD-dependent and haemin-independent species (Tables 4 and 15). *Haemophilus segnis* in many ways resembles *H. parasuis* (Table 15). The main features distinguishing the two species are the significant difference in the GC content of their DNA, the failure of *H. segnis* to produce α -fucosidase, and its negligible fermentative activity. Current studies have shown that *H. segnis* is frequently isolated from dental plaques, and the normal habitat of the organism is probably the human oral cavity (Kilian & Schiøtt, 1975).

Haemophilus parasuis. *Haemophilus* isolated from swine pose several problems of taxonomy and nomenclature. *Haemophilus influenzae suis* Lewis and Shope, 1931, is the name originally proposed for an organism isolated from American swine influenza and probably identical to the *Bacterium influenzae suis* described by Köbe (1933). *Haemophilus influenzae suis* was described as dependent on both X- and V-factors. It has been stressed that it closely resembles *H. influenzae* and, apart from a more feeble growth, is indistinguishable from indole-negative strains of the latter species (Lewis & Shope, 1931; Zinnemann, 1960). The organism is now usually referred to as *H. suis* in the literature, and because trinomial names are invalid the Subcommittee on the Taxonomy of *Haemophilus* has indicated that it prefers this epithet to the one originally proposed (Zinnemann, 1971). In *Bergey's Manual of Determinative Bacteriology* (1974) it is listed as *Haemophilus suis* Hauduroy, Ehringer, Urbain, Guillot and Magrou, 1937.

The pathogenicity of *H. suis* has been discussed in a number of recent reports, and it seems clear that it is the causative agent of porcine polyserositis, polyarthritis and meningitis (Glässer's disease), bronchitis, rhinitis and nephritis (Hjärre & Wramby, 1943; Bakos, Nilsson & Thal, 1952; Braend & Flatla, 1954*a*), whereas its aetiological role in swine influenza is variable and only secondary (Hjärre, Dinter & Bakos, 1952). Although most authors state that the implicated organisms are identical to *H. suis*, it has unfortunately been the habit not to determine the exact growth requirements. However, during the past decade it has become clear that the overwhelming majority of strains named *H. suis* are haemin-independent, but otherwise agreeing in the characters described by Lewis & Shope (1931) (Leidy, Hahn & Alexander, 1956; Biberstein *et al.*, 1963; Kilian, 1974). This finding stimulated Biberstein & White (1969) to propose the establishment of a new species *H. parasuis* with otherwise identical properties. Considering the marked difficulties associated with an exact determination of the haemin requirement and the complex nature of the growth requirements of *H. suis* (Shope, 1964), it is likely that the strains described by Lewis & Shope (1931) in fact were haemin-independent. This assumption is justified by the fact that two of the *H. suis* strains included in the present study (HK424 and HK425) originated from Shope himself, and that these two strains, like all other strains of porcine origin, possessed the ability to carry out porphyrin synthesis. This is also in agreement with the findings of Matthews & Pattison (1961). None of the strains of *H. suis* studied by them, including three strains received from Shope and four strains from Bakos, was haemin-dependent. Although it is thus highly likely that *H. suis* is the correct name for these haemin-independent strains, the name *H. parasuis* Biberstein & White, 1969 has been tentatively adopted here because the prefix of the epithet of that name is in agreement with the usual convention in naming *Haemophilus* species.

Two other strains included in the study were isolated from pneumonia in pigs and labelled *H. parasuis* on receipt. They grew better on all agar plate media than the strains assigned to *H. parasuis*, provided they were incubated with additional CO₂. Bakos *et al.* (1952) and Braend & Flatla (1954*b*) noted differences in the growth intensities of strains isolated from swine, which had no correlation with other properties. However, the two strains were also separated from the strains assigned to *H. parasuis* by several biochemical features. This is in agreement with the rather low similarity found by Sneath & Johnson (1973) between these strains and strains of *H. parasuis*. It is possible that the two strains represent a separate species, together with the three very similar strains isolated from fowls. All five strains have received the tentative label '*Haemophilus*, taxon C'.

Haemophilus pleuropneumoniae. The specific name *Haemophilus pleuropneumoniae* was proposed by Shope (1964) for a NAD-dependent organism causing extensive lobar pneumonia with accompanying fibrinous pleuritis in swine. The disease is highly contagious under experimental conditions and the fatality rate approaches 50 %. This marked virulence of *H. pleuropneumoniae* as a respiratory pathogen contrasts with the relatively mild pathogenicity of *H. suis* (Shope, 1964; Little & Harding, 1971). The organism is closely related to *H. parasuis*, as indicated by interspecific transformation experiments (White *et al.*, 1964), and it is probably identical to the causal agent of a septicaemic disease of swine briefly described by Biberstein & Cameron (1961) and identified as *H. parahaemolyticus* by Olander (1963). Nicolet (1968) demonstrated many similarities between *H. pleuropneumoniae* and *H. parahaemolyticus* and proposed the use of the latter name which has priority. This proposal has recently been adopted by the Subcommittee (Zinnemann, 1971) and in *Bergey's Manual of Determinative Bacteriology* (1974). However, there are many reasons for retaining a separate species for the virulent organism isolated from swine. It can be distinguished from

β -haemolytic NAD-dependent haemophili of human origin by several phenotypic differences (Table 13). The GC content of its DNA is significantly higher than that of human isolates ($P < 0.01$), and the two species possess a marked difference in their pathogenicity for swine. Four of the β -haemolytic human strains assigned to biotypes II and III of *H. parainfluenzae* were administered intranasally in large doses to piglets without causing any signs of disease, in contrast to the striking pathogenicity of strains of *H. pleuropneumoniae* even when inoculated in small numbers (Dr R. Nielsen, personal communication). Finally, haemolysis is not a distinctive taxonomic character within the genus *Haemophilus*. Shope (1964) in his description of *H. pleuropneumoniae* did not mention any haemolysis on blood agar plates and Nicolet (1970) noted a variable haemolytic activity. It is thus possible, as judged from the descriptions, that the causal agents of reported cases of porcine pneumonia and meningitis identified as *H. parainfluenzae* have been non-haemolytic strains of *H. pleuropneumoniae* (Matthews & Pattison, 1961; Radostits, Ruhnke & Losos, 1963). Although it is evident that *H. pleuropneumoniae* causes other infections in addition to pleuropneumonia, this is not a valid reason for changing the name. The correct name for the organism should therefore be *H. pleuropneumoniae* Shope, 1964.

The taxonomic and nomenclatural problems of porcine haemophili are far from resolved. As indicated by the present study, it is possible to recognize at least one haemolytic and two non-haemolytic NAD-dependent and haemin-independent taxa, and to this may probably be added a NAD- and haemin-dependent taxon (Biberstein *et al.*, 1963). Haemophili constitute a part of the normal respiratory flora of most swine (Radtke, 1938; Harris, Ross & Switzer, 1969; Woods *et al.*, 1972), and an extensive study of pathogenic strains as well as strains from normal respiratory flora is much needed to clarify the taxonomic situation.

Haemophilus paragallinarum. Haemophili isolated from fowls pose problems similar to those described for porcine haemophili.

Information on the growth requirements of the fowl-coryza bacillus *H. gallinarum* Delaplane, Erwin & Stuart, 1934 is conflicting. Most of the early descriptions of the organism state that it is haemophilic (de Blicke, 1932; Schalm & Beach, 1934; Eliot & Lewis, 1934; Delaplane *et al.*, 1934), but McGaughey (1932) was unable to show any dependence on the X-factor in his strains. A distinct need for haemin has only been convincingly demonstrated by Schalm & Beach (1936) and Delaplane, Erwin & Stuart (1938) who also showed an obligate requirement for NaCl. Several reports have been concerned with avian haemophili, but in none of these studies were haemin-dependent strains found (Page, 1962; Roberts, Hanson & Timms, 1964; Hinz, 1973). However, it is evident from these studies that several types of haemophili can be isolated from fowls, as already suggested by McGaughey (1932) and Nelson (1933). Biberstein & White (1969) proposed the name *H. paragallinarum* for haemin-independent haemophili with characters otherwise identical to *H. gallinarum*.

Only five strains isolated from fowls were included in the present study. All the strains required NAD only, but it was possible to recognize two groups among them. Two of the three strains, described together with the previously mentioned unnamed NAD-dependent strains from swine, were dependent on CO₂. This is in agreement with the character described for *H. gallinarum* (Schalm & Beach, 1934; Eliot & Lewis, 1934; Gregory, 1944). The abundant growth of these strains, the yellow pigmentation and the fermentation reactions are, however, more reminiscent of the non-pathogenic H-strains of McGaughey (1932) or the group II strains of Hinz (1973) and the taxonomic position of these strains cannot be established at present.

The two remaining strains were independent of CO₂ but grew with rather small colonies reminiscent of *H. gallinarum*. Page (1962), Roberts *et al.* (1964) and Hinz (1973) state that

the pathogenic *H. gallinarum* is characterized by a negative catalase reaction. Both of the strains here were catalase positive. As both strains were NAD-dependent only and one of the strains is an authentic strain of *H. paragallinarum* Biberstein & White (1969), they have both been assigned to this species. As with haemophili from swine, more extensive studies are needed to clarify the situation.

Haemophilus piscium. This species is usually considered the only *Haemophilus* species with no requirement for haemin or NAD, but with a need for diphosphothiamine or adenosine triphosphate (Griffin, 1951, 1952). The present study shows that the species differs from the type species *H. influenzae* by several other significant properties. The failure of *H. piscium* to reduce nitrate, to produce alkaline phosphatase and to grow at 37 °C, together with the high GC content of its DNA, indicate that there is little justification for its retention as a species of *Haemophilus*. The appropriate taxonomic position of the organism cannot be established at present. It shares many of the characters of *Francisella tularensis*, among them the requirement for ATP (Tamura & Fleming, 1949), but the DNA base composition of this species is very different from that of *H. piscium*.

Ecology and pathogenicity

Haemophili are parasites of the mucous membranes of man, and appear as a constituent of the normal microbial flora of the upper respiratory tract and, to a lesser extent, the mucous membranes of the genital organs. The same seems to be true for swine and fowls and probably also for a large variety of other animal species such as cats (Rivers & Bayne-Jones, 1923), rats (Wolff, 1903; Harr, Tinsley & Weswig, 1969), mice (Kairies & Schwartz, 1936), deer (Diernhofer, 1949), sheep (Mitchell, 1925), dogs, guinea-pigs (Kristensen, 1922), ferrets (Kairies, 1935) and monkeys (Kilian, unpublished results). Detailed information on the occurrence in many of these animal species is not available, however, and many of the haemophili isolated from these animals are not well known or described.

The human upper respiratory tract provides at least two different ecosystems as regards haemophili. The pharynx gives shelter to both haemin-dependent and -independent species (Kilian *et al.*, 1972), whereas haemin-independent species are almost the only species which can be isolated from the oral cavity (Sims, 1970; Kilian & Schiøtt, 1975). These species include *H. parainfluenzae*, *H. paraphrophilus* and *H. segnis*, of which the latter is present mainly in dental plaques (Kilian & Schiøtt, 1975). *Haemophilus aphrophilus* also occurs in dental plaques, although it is not invariably found, or at least not in detectable numbers (Kraut *et al.*, 1972).

Haemophilus influenzae is by far the most important of the *Haemophilus* species in human pathology and its pathogenic potentialities for humans are probably closely related to the presence of a capsule. Strains cultivated from patients with meningitis, epiglottitis or bacteraemia can usually be differentiated from non-pathogenic strains by their capsule production. Non-capsulated strains, on the other hand, can often be isolated from chronic infections, but it is seldom possible to assign a primary pathogenic role to such strains.

The recognition of at least five biotypes within *H. influenzae*, and the correlation demonstrated between these biotypes and the sources of the strains assigned to them (Table 18), may indicate that strains producing pyogenic infections are more specific than hitherto assumed. However, the aetiological significance of a number of the strains in the present study, especially those isolated from chronic infections including infections of the respiratory tract, cannot be proved.

Biotype I of *H. influenzae*, which encompassed nearly all strains from meningitis, epiglottitis and blood cultures, included only a few of the strains from other sources. Nine of

the 83 *Haemophilus* strains which originated from healthy upper respiratory tracts belong to this biotype. Two of these strains were capsulated strains of serotype b and were probably potential pathogens. Eight strains isolated from meningitis were assigned to other biotypes than type I. Two of these strains, however, were atypical, being non-capsulated, and all but one of the remaining strains were old stock strains. As the differentiation of the five biotypes rests on very few characters, it is possible that these stock strains represent mutants of biotype I. Further characterization of the biotypes is needed to answer this question.

Otitis media is often present in *H. influenzae* meningitis as in 50 (39 %) of the 128 cases of *H. influenzae* serotype b meningitis reviewed by Koch & Carson (1955). None of the 29 strains from otitis media included in the present study were associated with meningitis. Most of these strains belonged to different biotypes from most of the meningitis strains, and only two of the strains were capsulated. The predominance of non-capsulated strains in otitis media not associated with meningitis agrees with the observations of Harding *et al.* (1973) and Schneerson & Robbins (1973).

Most strains isolated from conjunctivitis belonged to biotypes II and III of *H. influenzae*, but several other taxa were represented. It is possible that some of the strains, as for example the strains of *H. parainfluenzae*, had been part of a transient contamination of the conjunctivae with no aetiological significance.

A proportion of the strains from conjunctivitis possessed haemagglutinating ability. This property was almost exclusive to strains isolated from the eye, but was not restricted to strains dependent on both NAD and haemin. Pedersen, Frøholm & Bøvre (1972) showed a connexion between fimbriation of *Moraxella bovis* and its ability to colonize the bovine conjunctivae and to produce keratoconjunctivitis. A positive correlation between haemagglutination and virulence has been demonstrated in *Bordetella pertussis* (Keogh & North, 1948) and between haemagglutination and fimbriation in Enterobacteriae (Duguid *et al.*, 1955), *Vibrio* sp. (Tweedy & Park, 1968) and *Bacteroides melaninogenicus* (Okuda & Takazoe, 1974). The true nature of haemagglutinin in *Haemophilus* strains is unknown, but it might be related to fimbriation and/or to the ability to colonize the conjunctivae.

The pathogenic potential of the species *H. parainfluenzae*, *H. paraphrophilus* and *H. aphrophilus* seems to be low as they only produce infections under special circumstances. The oral cavity is often a likely source of the aetiological organisms in such infections, which include endocarditis, dental abscesses, jaw infections and infections following human bites or finger sucking (Miles & Gray, 1938; Zinnemann *et al.*, 1968; Sims, 1970; Sutter & Finegold, 1970). In most other infections where these species have been reported as causal agents, it is usually impossible to prove an aetiological connexion. Reports on *H. parainfluenzae* as a frequent cause of meningitis, without any attempt to determine the properties of the strains involved except for an inadequate determination of growth requirements (Hable, Logan & Washington, 1971), should be regarded with suspicion.

The taxonomic situation of the genus Haemophilus

The relationship of the genus *Haemophilus* to other genera which have been included in the family *Brucellaceae*, or its predecessor *Parvobacteriaceae*, has not been considered in this study. These problems were recently studied by Johnson & Sneath (1973) and Sneath & Johnson (1973), who demonstrated a close phenetic relationship between the genera *Haemophilus*, *Pasteurella* and *Actinobacillus*, which on the other hand are well separated from the genera *Brucella*, *Bordetella* and *Moraxella*. These studies did not include strains of *A. actinomycetemcomitans*. The taxonomic position of this species is highly questionable. In *Bergey's Manual of Determinative Bacteriology* (1974) it is excluded from the genus

Actinobacillus and listed as *species incertae sedis*. As demonstrated previously, the species has a close phenetic similarity to *H. aphrophilus* and the question of its taxonomic position is connected with the taxonomic position of this species, and possibly *H. paraphrophilus* as well. These problems have considerable importance to the definition of the genus *Haemophilus*. The preservation of *H. aphrophilus* as a species and the inclusion of *A. actinomycetemcomitans* as a species in the genus *Haemophilus* would require a revision of the definition put forward by the Subcommittee on the Taxonomy of *Haemophilus* (Zinnemann, 1967). Such a revision would imply the possible affiliation with the genus *Haemophilus* of a number of bacteria hitherto excluded. Further studies are required before any such far-reaching decisions are taken.

I am grateful to Mrs Käthe Wedel Christensen and Mrs Lene Cramer for their skilful technical assistance throughout the course of the work and to Mrs Karen Helbo for preparing the typescript.

Many scientists generously contributed strains from their own collections. Particularly valuable material was received from Dr H. Lautrop and Dr C. H. Mordhorst (Statens Seruminstitut, Copenhagen, Denmark), Dr P. Bülow (Statens Seruminstitut, Århus, Denmark), Dr W. Frederiksen (Statens Seruminstitut, Ålborg, Denmark), Dr C. R. Dawson (Francis I Proctor Foundation for Ophthalmology, San Francisco, California), Dr Ragnhild Nielsen (Statens Veterinære Serumlaboratorium, Copenhagen), and Dr W. Sims (Royal Dental Hospital, London). The advice and encouragement of Dr Lautrop during all phases of the study is gratefully acknowledged. Dr C. Rindom Schjøtt, Dr H. S. Bleeg and Professor K. Zinnemann have given me much valuable advice and moral support, and Dr A. Leth Bak and Dr C. Christiansen (University of Århus) have kindly placed at my disposal the apparatus used for determining DNA base compositions. Dr A. Thylstrup (Royal Dental College, Århus) is thanked for statistical help; Dr V. Aagaard-Hansen (Jydsk Teknologisk Institut) kindly performed the gas chromatographic analysis.

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