HAEMIN AND NICOTINAMIDE ADENINE DINUCLEOTIDE REQUIREMENTS OF *HAEMOPHILUS INFLUENZAE* AND *HAEMOPHILUS PARAINFLUENZAE*

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PLATE XIV

HAEMOPHILUS INFLUENZAE requires haemin and nicotinamide adenine dinucleotide (NAD) for growth but *H. parainfluenzae* requires NAD only, and it is essentially on these nutritional characters that the two species are distinguished (Pittman, 1957). In practice, however, the determination of these requirements may be unreliable because basal media may contain traces of haemin (Zinnemann, 1960). *H. influenzae* grown on such media might appear to have no haemin requirement and be erroneously identified as *H. parainfluenzae*; also, the haemin present in heavy inocula could lead to the same conclusion (Biberstein and Gills, 1961; Parker and Hoeprich, 1962; Koser, 1968; Klein and Blazevic, 1969; Evans and Smith, 1972; Michaels and Phillips, 1972).

It has been suggested (Smith, Hale and O'Callaghan, 1953; Biberstein and Gills, 1961) that a sharp division into two distinct species may be an oversimplification and that *H. parainfluenzae* may merely represent one end of a wide series of *H. influenzae* strains ranging from those without the ability to synthesise haemin to those capable of growth in haemin-free media. Turk and May (1967) also consider that there is no point in distinguishing between the non-encapsulated strains of the two species and regard as rare strains that do not require some haemin for growth.

Hitherto, different media and techniques have been employed to measure haemin and NAD requirements for growth. One apparently significant variation was the size of the inoculum, which ranged from single cells on agar media to as high as 10^7 cells for cultures in fluid media (Gilder and Granick, 1948; Brumfitt, 1959; Butler, 1962; Parker and Hoeprich, 1962).

In this study an attempt has been made to clarify the position by measuring the haemin and NAD requirements of strains of *H. influenzae* and *H. parainfluenzae* on a medium considered to be free of haemin and employing very small inocula.

MATERIALS AND METHODS

Media. The basal medium used throughout contained (w/v) Proteose Peptone Broth (Difco) 2%, NaCl 0.6%, sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4 + \text{H}_2\text{O}$) 0.01% (Gilder and Granick, 1948), and glucose 0.2%, supplemented with thiamin hydrochloride 1 µg per ml and sodium

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oleate 4.8 μg per ml (Evans and Smith, 1972), and solidified with Oxoid Ionagar no. 2 1.2% (w/v). A clear agar similar to Levinthal's agar, referred to as X-V agar, contained in addition to the above ingredients, fresh yeast extract 1% (v/v) and lysed horse-blood 0.25% (v/v). For fluid cultures, the preparation of proteose peptone differed from the basal medium in containing yeast extract 1% (v/v) and haemin 20 μg per ml but no dithionite or thiamin. Proteose peptone broth used for washing and suspending the cells contained (w/v) only Proteose Peptone 2% and NaCl 0.6%. Chocolate agar (Cruickshank, 1965) was prepared from the basal medium with the addition of 7.5% of defibrinated horse-blood. Sterilisation of the basal medium was at 10 lbs per in.² (0.7 kg per cm²) steam pressure for 20 min., before glucose or supplements were added.

Organisms. All strains were Gram-negative rods, non-haemolytic on horse-blood agar, and were from type-culture collections or freshly isolated from cerebrospinal fluid, sputum, throat, and ear swabs cultured on X-V agar which usually contained 2.5 units of bacitracin per ml as a selective agent (Crawford, Barden and Kirkman, 1969; Hovig and Aandahl, 1969). Haemin and NAD requirements were determined by a satellitism test on the basal agar medium with and without haemin and with an inoculum standardised to give discrete colonies; a paper disk containing 15 μg of NAD was placed on the surface of each seeded plate (Smith and Evans, 1972). Encapsulated strains of *H. influenzae* were also typed by capsular swelling with *H. influenzae* antisera a-f (Burroughs Wellcome). Of 50 strains of *H. influenzae*, 43 were freshly isolated including 14 encapsulated of type b, and seven were reference strains, no. NCTC4560 (unencapsulated), no. NCTC8465 (type a), no. NCTC7279 (type b), no. NCTC8469 (type c), no. NCTC8470 (type d), no. NCTC10479 (type e), and no. NCTC8473 (type f). Thirty strains of *H. parainfluenzae* included 29 freshly isolated strains of which three required oleate and three thiamin, and a reference strain no. NCTC10665.

Chemicals and growth factors. Aqueous stock solutions of NAD (β NAD, Sigma Chemical Co. Ltd) and haemin (Koch-Light), as recommended by Turk and May, (1967), each contained 2000 μg per ml; thiamin hydrochloride (E. Merck) 10,000 μg per ml, sodium oleate (Sigma Chemical Co. Ltd) 12,000 μg per ml and glucose (Univar, Ajax Chemicals Ltd) 20% (w/v) were prepared in glass-distilled water; solutions were sterilised by filtration through a 0.45 μ Swinnex Millipore filter unit, and were added to the medium at 53°C. Potassium nitrate (KNO₃, Univar, Ajax Chemicals Ltd) was included at a final concentration of 0.1% (Gilder and Granick, 1948). Yeast extract was prepared from fresh baker's yeasts (Lwoff and Lwoff, 1937), and stored deep frozen.

Inocula for determination of quantitative haemin and NAD requirements. Overnight chocolate-agar cultures were used to seed 10-ml amounts of broth in 30 ml screw-capped containers which were shaken in a water bath for 6 hours at 37°C. Organisms were recovered by centrifugation and washed four times in proteose peptone broth before being resuspended in the same medium. Ten-fold dilutions of these washed cell suspensions were seeded on duplicate plates by a multiple inoculating device (Steers, Foltz and Graves, 1959); the volume of inocula was 4 μl and the smallest inoculum contained 1-20 cells. In later experiments, it was found that the washing process was unnecessary and that inocula could be prepared by suspending one colony from a chocolate-agar culture in 2.5 ml of proteose peptone broth; the suspension thus obtained was diluted 1 in 10,000 and contained 1-20 viable units in 4 μl .

Culture conditions. All aerobic cultures were incubated for 24 hours in an atmosphere of 5% CO₂ in air (v/v) at 37°C. Anaerobic cultures were incubated for 48 hours in an atmosphere of hydrogen with 5% CO₂ (v/v), in a McIntosh and Fildes jar with a palladium chloride cold-catalyst (Baird and Tatlock); a Lucas, semi-solid, anaerobic indicator (Willis, 1964) was used routinely and the efficiency of the anaerobic system was checked regularly with a blood-agar culture of *Clostridium tetani*.

Estimation of growth on solid media. The main criterion of growth was that the colonies developing were uniform in number and size, and were comparable in number with those on control plates of chocolate agar. Colony sizes were measured with a microscope with a $\times 3.5$ lens and a micrometer eyepiece, or a measuring magnifier.

RESULTS

The NAD requirement of H. influenzae and H. parainfluenzae in air with 5% CO₂

The amount of NAD required for the growth of 50 strains of *H. influenzae* and 30 strains of *H. parainfluenzae* was determined on the complete basal medium containing 20 µg haemin per ml and with an inoculum of 1–20 cells. For the growth of all 80 strains of both species the least amount of NAD required was 5 µg per ml; colony counts were comparable with those on chocolate agar as were the colony sizes, which averaged 1.5 mm diameter for

TABLE

The effect of NAD concentration on the colony size of representative strains of Haemophilus influenzae and H. parainfluenzae from an inoculum of 1–20 cells

Medium	Colony diameter (mm) of	
	<i>H. influenzae</i>	<i>H. parainfluenzae</i>
Basal agar + haemin 20.0 µg per ml	0	0
+ NAD 0.008 µg per ml	0	0
0.04 µg per ml	0.1–0.5	0
0.2 µg per ml	1.0	0
1.0 µg per ml	1.5	0.5
5.0 µg per ml	1.5	1.0
25.0 µg per ml	1.5	1.0
Chocolate agar	1.5	1.0

unencapsulated strains and 2.0 mm for encapsulated strains of *H. influenzae* and 1.0 mm for *H. parainfluenzae*. Three unencapsulated strains of *H. influenzae* had colonies of small size, ranging from 0.2 to 0.7 mm; of these one was CO₂-dependent and the other two also grew poorly on chocolate agar.

With the exception of three oleate-requiring strains of *H. parainfluenzae*, which required NAD 25 µg per ml for maximal growth, appreciable difference in colonial size or number was not observed with concentrations of NAD over 5 µg per ml. The most common effect of the concentration of NAD on the colony size of a typical strain of each of the two species is recorded in the table.

With a decrease in NAD concentration to 1.0 µg per ml, the size and numbers of colonies of *H. influenzae* strains remained unchanged. Of the 30 strains of *H. parainfluenzae* however, 14 either failed to grow at all, or grew with small and variable-sized colonies averaging 0.5 mm diameter. Further reductions of the NAD concentration to 0.2 µg per ml resulted in the growth of only two strains of *H. parainfluenzae*, one appearing as tiny colonies of 0.2 mm the other as colonies of 0.8 mm diameter. All 50 strains of *H. influenzae* continued to grow from small inocula with NAD 0.2 µg per ml, but colony diameters were slightly reduced (0.8–1.2 mm).

Even at a lower concentration of NAD (0.04 µg per ml) at least half of the *H. influenzae* strains continued to grow from inocula of 1–20 cells although

colony sizes were further reduced and variable (0.2–0.5 mm); no strains of *H. parainfluenzae* gave visible growth at this concentration. With an NAD concentration of 0.008 μg per ml there was no growth of any of the strains of either species, even with a ten-fold increase in inoculum size.

Of the 30 strains of *H. parainfluenzae*, three did not grow in the absence of oleate, but when this substance was present, or on chocolate agar, these strains produced colonies of a larger diameter, approximately 2.0 mm, compared with the 1.0 mm of the other strains. Three thiamin-requiring strains produced colonies of the smaller size on the complete basal medium.

Haemin requirement of H. influenzae in air with 5% CO₂

The 50 *H. influenzae* strains grew on the basal medium containing haemin 20 μg and NAD 1 μg per ml from inocula of 1–20 cells. The size and number of the colonies were similar to those of colonies on chocolate agar; no strain grew in the absence of haemin. Further examination of the haemin requirements of 10 strains showed that the minimum requirement for optimum growth ranged between 2 and 10 μg per ml; at concentrations of less than 2 μg per ml the colony size of all strains was progressively diminished until at a concentration of 0.05 μg per ml there was no growth; with larger inocula of 100–1000 cells, some strains showed visible but slight growth at this concentration of haemin.

Growth of H. influenzae in hydrogen with 5% CO₂

Four strains of *H. influenzae*, including two encapsulated type-b strains, were grown anaerobically on the basal medium containing NAD but no haemin. After 10 serial subcultures, with inocula of 1–20 cells on each occasion, the numbers of colonies of each strain were similar to those on chocolate agar incubated in air with 5% CO₂ but the colony size was smaller and was 0.7–1.0 mm after 48 hours' incubation. Glucose was essential for full growth of the inoculum and in its absence the number and size of colonies developing was diminished. Six other strains grew anaerobically on three separate occasions, also from the same small inocula.

The separate addition of either haemin or potassium nitrate to the basal medium containing NAD had no effect but the addition of both haemin and nitrate resulted in increased colony sizes, which averaged 1.2 mm at 48 hours.

Growth of H. parainfluenzae in hydrogen with 5% CO₂

Thirty strains of *H. parainfluenzae* were also cultured anaerobically with NAD 5 μg per ml on the basal medium. Potassium nitrate was essential for some strains and enhanced the growth of the remainder which, in the absence of nitrate, produced colonies small and variable in size.

DISCUSSION

H. influenzae is characterised by its morphology and requirement for X and V factors when grown on nutrient media. The identity of X factor as haemin

GROWTH REQUIREMENTS OF *HAEMOPHILUS*

FIGURE.—Growth after 24 hours on plates containing basal medium+haemin $20\text{ }\mu\text{g}$ per ml, with the addition of NAD (above) $0.2\text{ }\mu\text{g}$ per ml and (below) $5.0\text{ }\mu\text{g}$ per ml; upper halves of plates, two strains of *Haemophilus influenzae*; lower halves, two strains of *H. parainfluenzae*. Three 10-fold dilutions of each culture were used as inocula.

was established by Fildes (1921). *H. parainfluenzae* was subsequently recognised as a distinct species by its requirement of V factor only (Rivers, 1922) and this was identified by Lwoff and Lwoff (1937) as co-enzyme I or NAD. The amounts of haemin and NAD required by the two species have been estimated in several studies under different conditions, including different basal nutrient media, either as broths or solidified with agar and from inocula that ranged from 10^1 to 10^7 cells.

The amount of haemin required for growth of *H. influenzae* has been stated to be 0.05–0.3 μg per ml by Lascelles in a review (1962); chocolate agar, a traditional medium for the growth of *H. influenzae*, usually contains more than 500 μg per ml. Parker and Hoeprich (1962), using a fluid medium and an inoculum of 10^3 cells, found that at least 10 μg per ml was required. Gilder and Granick (1948) determined the requirement as 0.01–0.1 μg per ml with an inoculum of 10^7 cells; Biberstein and Spencer (1962) found in some instances a minimal requirement of 0.01 μg per ml and certain strains grew even in the absence of added haemin, but they considered that amounts adequate for growth might have been present in the basal medium and the inoculum. On a solid medium with a small inoculum the haemin requirement was determined as 2 μg per ml (Brumfitt, 1959). Defined media for the growth of haemophilus species contain approximately 10 μg per ml (Wolin, 1963; Herriott *et al.*, 1970). The results of the present study indicate that the haemin requirement of *H. influenzae* strains is between 2 and 10 μg per ml and do not support the view, expressed by Smith, Hale and O'Callaghan (1953) and by Turk and May (1967), that strains differ widely in this respect. At concentrations of haemin of less than 2 μg per ml, colonies were small and reduced in number, and growth was not evident at concentrations of less than 0.05 μg per ml. The varying amounts of haemin reported as minimal requirements may therefore be attributable to differences in the experimental conditions employed.

H. influenzae grew anaerobically without haemin. Four strains continued to grow anaerobically after 10 serial subcultures from small inocula without added haemin on the basal medium containing glucose. The addition of haemin to the medium was associated with an increase of growth only if nitrate was also present as a hydrogen acceptor.

None of the strains of *H. parainfluenzae* required haemin, but it was confirmed that this substance acted as a reducing agent replaceable by dithionite, and therefore appeared to be a requirement for some strains (Gilder and Granick, 1948). Under anaerobic conditions, *H. parainfluenzae*, unlike *H. influenzae*, grew poorly on the basal medium, but growth was greatly enhanced by the addition of nitrate. This accords with the findings by White and Smith (1962) that anaerobic growth of *H. parainfluenzae* was nitrate-dependent; they also showed that under these conditions the cells contained large amounts of cytochrome a_1 oxidisable by nitrate. In contrast, cytochrome was not detected in the cells of *H. influenzae* grown anaerobically without nitrate (White, 1963).

There appear to have been few determinations of the minimum requirements of NAD for growth. Most growth media for *Haemophilus* species include yeast extract—which contains approximately 150 μg of NAD per ml—or purified

NAD 1–3 μg per ml (Butler, 1962; Biberstein and Spencer, 1962; Wolin, 1963). Parker and Hoeprich (1962), with a fluid medium and an inoculum of 10^3 cells, obtained maximal growth of a strain of *H. influenzae* type b with an NAD concentration of 0.5 μg per ml. Lwoff and Lwoff (1937), in their analysis of the physiological role of factor V, described a strain of *H. parainfluenzae* that required as little as 0.004 μg per ml when grown in proteose peptone broth; we have not encountered such a strain. Rather we have found that strains of *H. influenzae* grow at a lower concentration of NAD than is required for *H. parainfluenzae*. All 50 strains of *H. influenzae* grew from small inocula on the basal medium containing NAD 0.2 μg per ml, the size and numbers of colonies developing being comparable to those on control plates of chocolate agar. For *H. parainfluenzae*, a concentration of 5 μg per ml was required to support growth of all 30 strains and only two strains showed any growth with NAD 0.2 μg per ml. About half of the strains of *H. influenzae* showed growth at a concentration of NAD as low as 0.04 μg per ml but no strain of *H. parainfluenzae* grew at this concentration.

In conclusion, we found no evidence that strains of the two species represent a gradient in capacity to synthesise porphyrins. The two species are distinguishable on the basis of the requirement for haemin by *H. influenzae*. *H. parainfluenzae* does not require haemin and, furthermore, probably represents a more heterogenous group and includes strains that require thiamin or oleate (Butler, 1962; Evans and Smith, 1972).

SUMMARY

Fifty strains of *Haemophilus influenzae* and 30 strains of *H. parainfluenzae* were clearly differentiated by the requirement of *H. influenzae* strains for haemin on a proteose-peptone basal medium and with an inoculum of 1–20 cells. No evidence was obtained for a wide range in haemin requirements; 10 strains of *H. influenzae* examined in detail required 2–10 μg haemin per ml. The two species were dissimilar also in the amount of NAD required; the strains of *H. influenzae* grew well in the presence of NAD 0.2–1.0 μg per ml, whereas most strains of *H. parainfluenzae* required 1.0–5.0 μg per ml and three strains required NAD 25 μg per ml for optimal colony size. Nitrate was essential for the anaerobic growth of *H. parainfluenzae* but not of *H. influenzae*.

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