A Haematin-Independent Mutant of *Haemophilus influenzae*

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SUMMARY

A mutant of *Haemophilus influenzae* has been isolated and found to be independent of haematin. Its microscopic morphology and growth properties were found to be similar to those of a strain of *H. parainfluenzae* and not to those of its parent. The use of non-synthetic media in assessing the haematin requirements of haemophilus strains is discussed.

INTRODUCTION

Organisms which have been given the generic name *Haemophilus* are grouped together because of their requirement for blood for growth in culture media. It was found that two growth factors present in blood were involved and these were termed the X and V factors. Fildes (1921) showed that the X factor could be replaced by haematin, and Lwoff & Lwoff (1936, 1937) identified the V factor as either di- or tri-phosphopyridine nucleotide. Within the group strains have been isolated from natural sources requiring both the X and V factors, and others needing only one of them. Those requiring both factors have been termed *Haemophilus influenzae* and those requiring only the V factor have been termed *H. parainfluenzae*. The microscopic appearance of these two subgroups also tends to differ. *H. influenzae* appears predominantly as very small cocco-bacilli 1–1.5 by 0.3–0.4 μ (Topley & Wilson's *Principles*, 1955), known as the 'typical' morphology, although the organism is pleomorphic and longer forms may occur, either as a small minority amongst the 'typical' cells, or as a majority under changed cultural conditions. On the other hand, *H. parainfluenzae* appears predominantly as well-defined rods. However, within the *H. influenzae* subgroup there is a continuous gradation as regards the degree of requirement for haematin since some strains require less haematin than others (Smith, Hale & O'Callaghan, 1953; Biberstein & Gills, 1961). The present paper describes some properties of a mutant of *H. influenzae*, produced in the laboratory and exhibiting complete independence of haematin.

MATERIALS AND METHODS

Organisms. The following organisms were used: *Haemophilus influenzae* (Rd) of Alexander & Leidy (1953), a rough mutant from a wild Pittman type d; *H. influenzae* (RdTr), a strain derived from Rd by Dr P. Schaeffer, resistant to streptomycin, erythromycin and novobiocin; and *H. parainfluenzae* (FID), isolated from a human throat by Dr H. E. Alexander; these will be referred to in the text as strains Rd, RdTr and FID. All strains were maintained on slopes of chocolate tryptone agar in cotton-wool plugged test tubes kept at 37°.

Tryptone agar (TA). Tryptone (Difco) was dissolved in glass-distilled water at 20 g. together with NaCl at 10 g./l. The pH was brought to about 3 with HCl and activated charcoal added to give a 0.2% (w/v) suspension. The mixture was allowed to stand with occasional shaking for 15 min. and then filtered. The filtrate was adjusted to pH 7.5 and agar (Davis) added to give a 2% (w/v) solution. The medium was stored at this stage. When required, the molten tryptone agar was dispensed in 20 ml. volumes into 25 ml. screw-capped bottles and cooled to 56° in a water bath. Half a millilitre of a 4% (w/v) solution of crystalline bovine albumin was then added and the mixture poured as plates.

Tryptone blood agar (TBA). The molten tryptone agar was dispensed in 20 ml. volumes into 25 ml. screw-capped bottles, cooled to 56° in a water bath, and oxalated horse blood added to give a 5% (v/v) suspension. The mixture was then poured as plates.

Chocolate tryptone agar (CTA). To the molten tryptone agar cooled to about 80° was added oxalated horse blood to give a 5% (v/v) suspension, and the mixture allowed to turn to a chocolate colour, with heating further in a water bath if required. The medium was then dispensed as plates or as slopes in cotton-wool plugged tubes.

Yeast extract solution. Yeast extract (Difco) was dissolved in glass-distilled water to make a 10% (w/v) solution, and sterilized by Seitz filtration.

Glucose-salt solution. This consisted of 5.0 g. glucose, 17.5 g. K_2HPO_4 , 5.0 g. KH_2PO_4 , 1.056 g. Na_3 citrate $5H_2O$, 0.25 g. $MgSO_4 \cdot 7H_2O$, and 2.5 g. $(NH_4)_2SO_4$ in 100 ml. glass-distilled water. The mixture was sterilized by autoclaving at 115° for 10 min.

Haematin solution. A stock solution of haematin (Roche Products Ltd.) was prepared as follows: 50 mg. haematin was mixed with 12.6 ml. 0.5M- Na_2HPO_4 , heated to dissolve, and 86 ml. glass-distilled water + 1.6 ml. M- KH_2PO_4 added. The solution was sterilized by autoclaving at 115° for 10–15 min.

Coenzyme I solution. Diphosphopyridine nucleotide (Sigma Chemical Co.) was dissolved in glass-distilled water at a concentration of 1 mg./ml., 0.06 ml. of this solution was used in 21 ml. medium, i.e. at a final DPN concentration of 0.3 mg./100 ml. medium.

Synthetic medium (SM). This was a chemically defined medium with the composition previously described (Butler, 1962).

Evaluation of growth requirements. Suspensions of the organisms were made in normal saline and a loopful spread over a segment of a plate of medium. After incubation at 37° for 24 and 40 hr., a visual assessment was made of the density and homogeneity of growth and of colony size.

Serological reactions. Antisera were prepared in rabbits by giving a course of injections of a suspension of organisms in physiological saline at an approximate concentration of 10^9 cells/ml. Two injections, 6 days apart, of 0.2 ml. of the suspension into the vein of the ear, were followed at 6-day intervals by three further injections of 0.4 ml. The rabbits were bled 5 days after the last injection and the serum separated and kept at 4° until tested.

Tube agglutination was carried out in 6 × 1 cm. glass test tubes. An initial serum dilution in saline of 1/10 was made, and from this serial doubling dilutions in saline were set up using 0.5 ml. volumes. To each tube of serum dilution was added an

equal volume of a saline suspension containing about 10^8 bacteria/ml. The range of final serum dilutions was 1/20 to 1/5,120. Each titration included a negative control consisting of a tube containing the bacterial suspension with an equal volume of saline only. All dilutions and controls were incubated for 18 hr. at 56° followed by 1 hr. at 4° . The presence of granular agglutination was then observed visually with the aid of a concave mirror. The titre of a serum was recorded as the reciprocal of the final dilution containing the least amount showing clear-cut agglutination.

Table 1. *Composition of medium for the isolation of strain Rd 1, the haematin-independent mutant of the Haemophilus influenzae strain Rd*

Substance	mg.	pH of stock solution	
Glycine	0.96	—	Dissolved in glass distilled H_2O to give total volume 6.0 ml.
DL-Alanine	0.96	—	
DL-Valine	2.88	6.9	
L-Leucine	2.88	6.9	
DL-Isoleucine	1.6	6.9	
DL-Serine	2.88	—	
DL-Threonine	1.44	—	
DL-Aspartic acid	2.88	6.9	
L-Glutamic acid	4.8	6.9	
L-Arginine HCl	0.96	6.9	
L-Lysine (HCl) ₂	2.88	—	
L-Cysteine HCl*	2.5	—	
DL-Methionine	1.44	—	
Glutathione*	5.0	—	
DL- β -Phenylalanine	1.44	6.9	
L-Tyrosine*	1.6	6.9	
L-Tryptophane*	0.48	6.9	
L-Histidine HCl	1.19	6.9	
L-Proline	2.88	—	
Crystalline bovine albumin*	40.0	—	
	ml.		
Yeast extract solution	1.0		
Glucose-salt solution	0.8		
Haematin solution	0.3		
Coenzyme I solution*	0.06		
2% (w/v) N.Z. (Davis) agar in glass distilled H_2O	13.0		

Except where indicated, constituents were sterilized by autoclaving at 115° for 10–15 min.

* Sterilized by Seitz filtration.

RESULTS

Production and isolation of the mutant Rd 1. During the early stages of development of the chemically defined medium (SM) for *Haemophilus influenzae* reported previously (Butler, 1962), a yeast-extract-containing medium (YEM) of the composition shown in Table 1 was used. Segments of plates of this medium were inoculated with strains Rd, RdTr and FID and spread over the surface. After 4 days incubation at 37° , strain Rd had given rise to several colonies, densely white in the centre and with a translucent rim; under them a white deposit appeared in the medium. The colonies were subcultured on to stock CTA and later streaked on to TA, TBA, TA + DPN, together with spot inocula of staphylococci, and also on to YEM and

CTA. On the two latter media the organisms grew along the full length of the streaks as small colonies down to the lowest dilution, but growth was potentiated around the staphylococci. On TA, satellitism was shown around the staphylococci but the colonies were small, and on TA + DPN good growth occurred over the whole inoculum. The strains RdTr, FID and the parent organism Rd, were also streaked on to these media (except YEM and CTA) for comparison. All three strains grew well on the blood medium, giving small colonies over the whole inocula, and satellitism. On TA, strain FID showed satellitism but neither strains Rd nor RdTr grew, and with added DPN, both strains FID and RdTr grew but no growth was obtained with strain Rd. These results are summarized in Table 2. It seemed clear that a mutant of strain Rd had been isolated which was less dependent on added haematin, and it was designated strain Rd1. It was subsequently maintained on slopes of CTA.

Table 2. *Comparison of the capacity of various media to support growth of strains Rd, RdTr, FID and the mutant Rd1*

Strain	1* TBA	2* TA	3* TA + DPN	4 CTA	5 YEM	6 SM + haematin (mg./100 ml. medium)			
						0	1.2	1.8	2.4
Mutant (Rd1)	G+S	S	G	G	G	G	G	G	G
Rd	G+S	O	O	—	—	O	G	G	G
RdTr	G+S	O	G	—	—	O	G	G	G
FID	G+S	S	G	—	—	G	G	G	G

* Media 1, 2 and 3 were streaked with *Staphylococcus aureus* to demonstrate satellitism.

G = growth

TBA = tryptone blood agar

S = satellitism

TA = tryptone agar

G+S = growth enhanced around staphylococci

CTA = chocolate tryptone agar

O = no growth

YEM = yeast extract medium

DPN = diphosphopyridine nucleotide

SM = synthetic medium

Haematin requirement. The haematin requirement was ascertained with the use of SM by varying the amount of haematin in that medium. The final concentrations of haematin used were 0, 1.2, 1.8 and 2.4 mg./100 ml. medium. All strains grew at concentrations down to 1.2 mg./100 ml., but only strains FID and Rd1 grew without haematin. The growth was equal to that on the haematin-containing plates. These results are also included in Table 2. It was concluded, therefore, that strain Rd1 was haematin-independent.

Microscopic morphology. Smears made from 24 hr. stock slopes of strains Rd, RdTr, FID and Rd1 were Gram stained using dilute carbol fuchsin as counter-stain. Strain Rd had the 'typical' appearance, being predominantly cocco-bacillary with a few long forms; strain RdTr was similar to its parent strain Rd. Strain FID had the 'atypical' appearance, with mainly well-defined rods and only a few, if any, other forms; and strain Rd1 resembled strain FID exactly.

Growth requirements. Table 3 summarizes the results obtained concerning the purine, pyrimidine, amino acid and mineral salt requirements of strain Rd1, obtained during the development of SM previously reported. The growth requirements of strains Rd, RdTr, and FID were ascertained at the same time and have been reported elsewhere (Butler, 1962). It will be noted that strain Rd1 has an

essential requirement for uracil, pyridoxine, isoleucine and valine. The uracil could be replaced by guanine + hypoxanthine. A reduction in the concentration of phosphates (not mentioned in Table 3) below the level normally included in SM resulted in reduced growth of strain Rd1. Strains Rd, RdTr and FID required albumin or polyvinyl alcohol in the medium and exhibited a peak of maximum growth when subjected to varying concentrations of these substances. Strain Rd1 behaved similarly, resembling strain FID in its behaviour with albumin, and strain RdTr in respect of polyvinyl alcohol. Sodium oleate could replace albumin or polyvinyl alcohol, as found for strains Rd, RdTr and FID.

Table 3. *Purine and pyrimidine, vitamin, amino acid and mineral salt requirements of the mutant Rd1*

Type of substance	Essential	Stimulatory	Not required
Purines and pyrimidines	Uracil (replaceable by guanine + hypoxanthine)	Adenine	Thymine, cytosine, hypoxanthine, guanine
Vitamins	Pyridoxine	Ca pantothenate, putrescine (aneurin in absence of Ca pantothenate)	Biotin, folic acid, aneurin, glutamine
Amino acids	Isoleucine, valine	Tryptophane, cysteine or glutathione, histidine, tyrosine, arginine, aspartic acid, glutamic acid, leucine, phenylalanine	Lysine, glutathione or cysteine, methionine, alanine, glycine, proline, threonine, serine
Mineral salts	ZnSO ₄	Na acetate, NaHCO ₃ , MgSO ₄ , FeSO ₄	CaCl ₂ , MnCl ₂

Repetition of isolation of the mutant

In view of the fact that the mutant designated Rd1 was isolated in the course of investigations in which the strains Rd, RdTr and FID were often inoculated on different segments of one plate of medium and the remarkable resemblance between the properties of strains Rd1 and FID, it was thought desirable to attempt another isolation of a similar mutant from the parent strain Rd. On repeating the experiment in the same way as in the original isolation, colonies were obtained in the segment inoculated with strain Rd, which, after first subculturing on to CTA, were found to be haematin-independent. Subculture *directly* from the YEM plate on to SM without haematin gave no growth, and on to TA with 'spot' inocula of staphylococci gave only barely detectable satellite growth. Colonies also appeared on the segment of the YEM plate inoculated with strain RdTr, but they did not cause a precipitation to occur in the medium below the colonies, nor were the organisms found to be haematin-independent.

Serological reactions. The titres obtained by tube agglutination of the cell suspension of the parent strain Rd and the mutant strain Rd1 with their antisera are shown in Table 4. Satisfactory homologous reactions were observed, strain Rd1 producing higher titres than did strain Rd. Cross-agglutination, however, was seen in only one direction. Cells of the parent strain Rd were agglutinated by the serum

of the mutant strain, but no agglutination was detected between cells of the mutant strain and the antiserum to the parent strain. When the heterologous cells in the cross-tests were removed by centrifugation and the *homologous* cells added to the supernatants, agglutination was obtained after further incubation, to the same titres as had already been found in the conventional homologous titrations, showing that the negative reaction (anti-Rd with Rd1 cells) was not due to the presence of a soluble inhibitor in the Rd1 bacillary suspension.

Table 4. *Titres of agglutination of cells of parent strain Rd and of haematin-independent mutant Rd1 by their antisera and by supernatants from mixtures of antisera with heterologous cells*

Cells	Antisera		Supernatants	
	Rd	Rd1	Rd*	Rd1†
Rd	320	320†	320	
Rd1	< 10*	2560		2560

*† After recording the titres obtained, the cells of the heterologous tests were removed by centrifugation and the supernatants mixed with the homologous cells and agglutination titres noted after further incubation.

Table 5. *Comparison of the properties of strain Rd1 with those of strains Rd and FID*

Predominantly FID-like properties	Predominantly Rd-like properties	Intermediate properties	Neither Rd- nor FID-like properties
Haematin independent	Stimulation by NaHCO ₃	Purine and pyrimidine requirements	Stimulation by Na acetate
Microscopic morphology			
Stimulation by amino acids on 'Zeocarb' treated yeast extract			
Vitamin requirements			
Amino acid requirements			
Behaviour with albumin			

Strain Rd = *Haemophilus influenzae* (rough from type d).
 Strain Rd1 = haematin-independent mutant of strain Rd.
 Strain FID = *H. parainfluenzae*.

DISCUSSION

The organism Rd1 is an example of a mutant, derived by artificial selection from a haematin-dependent *Haemophilus influenzae*, which has become completely haematin-independent. Such an organism would be classified as a strain of *H. parainfluenzae* according to the criteria commonly used in identifying members of the genus *Haemophilus*. The curious features of the isolation of the mutant were that its colonies appeared on a medium (YEM) which contained at least 0.7 mg. haematin per 100 ml. medium, but which was unable to maintain normal growth of the parent strain Rd, and that these colonies gave rise to a culture growing well on SM without haematin, and on other media lacking haematin, but only after subculture on a complete medium (CTA). On the other hand, no similar mutant was obtained by a direct method—i.e. by plating out large inocula of strain Rd on SM without haematin.

This procedure was carried out several times, since it formed part of the routine check for purity of the stock cultures of strain Rd. The inocula of this strain so tested were as large as, or even larger than, those from which the mutant was obtained on YEM. These findings suggest that there may have been a multiple-step process of mutation to haematin-independence, also involving a stage of growth on complete medium for phenotypic expression to occur; for example, the initial phase of selection on YEM may have been towards independence of some simpler constituent (e.g. adenine, which is required by the parent but not by the mutant), and after this change had become phenotypically expressed, haematin-independence became conferred on the organism. However, since the exact composition of the yeast extract was not determined, and in the absence of sufficient knowledge of the metabolic pathways involved, it is not possible to decide what may be the correct explanation of the phenomenon.

The results given in Table 2 with respect to the undefined media would suggest that strain RdTr might be haematin-independent also, since it grew on medium containing only tryptone agar + DPN, but it gave no growth on the defined medium without haematin. These facts emphasize the following points:

(1) The extent of haematin independence can only be determined by the use of a defined medium. The tryptone used above obviously contained a small (and almost certainly variable) amount of haematin or haematin-like compound. The presence of haematin or similar substances had been suspected in such media by others (e.g. Biberstein & Gills, 1961), and Gilder & Granick (1947) also reported the presence of haematin in yeast extract. It is pertinent to note, therefore, that all results referring to the need or otherwise of haematin obtained by using an undefined medium must be viewed with some reservation. For example, the reported haematin independence of *H. influenzae* growing anaerobically should be re-assessed. The finding of Gilder & Granick that haematin is still required for anaerobic growth but in smaller amounts is quite likely, but these authors used a proteose-peptone base which may well have contained some haematin so that the actual requirement could not have been determined.

(2) The haematin content of the tryptone was sufficient to allow growth of strain RdTr but not of strain Rd. This supports the view that there is a gradient of haematin requirement amongst the haematin-dependent strains (Smith *et al.* 1953; Biberstein & Gills, 1961). This may account for the unsuccessful attempts to isolate a haematin-independent mutant of strain RdTr using exactly the same conditions as for strain Rd. It may be that as strain RdTr appears normally to be able to grow with lower concentrations of haematin than strain Rd, the haematin content of the 'mutation medium' may have been too high to have a selective influence. However, this point was not further investigated.

It is interesting to compare the properties of strain Rd1 with those of its parent strain Rd and those of strain FID. Smears of growth of the same age taken from CTA slopes show predominantly well-defined rods for strain Rd1 and FID, while strain Rd is predominantly cocco-bacillary. On a medium containing Zeo-carb treated yeast extract (Butler, 1962) strain Rd would not grow, but strains Rd1 and FID gave reduced growth which was stimulated in both organisms by the addition of amino acids. The vitamin and amino acid requirements were exactly those of strain FID, both requiring pyridoxine, isoleucine, and valine as essential

metabolites, and both stimulated by the same vitamins and amino acids, whilst strain Rd showed no absolute requirement for any one vitamin or amino acid. The behaviour with albumin was similar to that of strain FID, although the behaviour with polyvinyl alcohol was similar to that of strain RdTr, the other mutant derived from the same parent. The purine and pyrimidine requirement was intermediate between strains Rd and FID. Strain Rd1 required uracil and was stimulated by adenine, although the uracil could be replaced by guanine + hypoxanthine. Strain Rd had an essential requirement for both uracil and adenine, whilst strain FID required guanine and was stimulated by uracil and adenine. Most of the mineral salt requirements were shared with both strains Rd and FID, with two exceptions, viz. NaHCO_3 stimulated strain Rd1, Rd (and RdTr) but not strain FID, and sodium acetate stimulated strain Rd1 only. These comparisons are summarized in Table 5 from which it can be seen that the properties of strain Rd1 are predominantly similar to those of strain FID. It is therefore tempting to suggest that as both strains Rd1 and FID require valine, it may be that this amino acid is required in the pathway leading to the synthesis of haematin or in an alternative pathway bypassing the need for haematin altogether. It may also be of interest to note that both strains Rd1 and FID are independent of adenine (required by strain Rd), although strain Rd1 retained its parental requirement for uracil.

It is necessary to consider the possibility of contamination by *Haemophilus parainfluenzae*:

(1) *Contamination of stock culture.* As stated above, the stock culture of strain Rd was maintained on slopes of CTA. These were subcultured twice weekly, and were periodically inoculated on to plates of TA and checked for satellitism, and also on to SM not containing haematin. No growth was obtained in either case. Stock cultures were never taken from plates also containing strain FID. It is also felt that a *Haemophilus parainfluenzae* contaminant would not require an initial subculture on CTA before the expression of its haematin-independence, since this character would already have been expressed.

(2) *Contamination of plate.* Colonies have been obtained on YEM plates inoculated with strain Rd, but not also inoculated with strain FID, which have resembled the strain Rd1. Again, it is felt that the comment made above concerning the lag in the expression of the haematin independence is relevant. Also it may be significant that the colonies showing haematin-independence always appeared on segments inoculated with strain Rd and not on those inoculated with strain RdTr.

No attempt was made to obtain the mutant from cultures grown from single-cell isolates of strain Rd: as pointed out by Hilson & Elek (1959), it is doubtful whether this procedure affords any significantly greater protection against contamination than conventional methods of obtaining and handling pure cultures.

The property of haematin-dependence is heavily relied on to distinguish between *Haemophilus influenzae* and *H. parainfluenzae*. The isolation of a haematin-independent mutant of *H. influenzae* shows that this character is not stable on a medium, which although unfavourable to the general growth of the strain used, was not deliberately devised for the selection of haematin-independent mutants. These results suggest that workers who are involved in the isolation, identification and maintenance of *H. influenzae* strains should take particular care not only that the medium used for their culture is complete, but also that the cultures should be

subjected to the least number of subcultures through various media. Such precautions should minimize the risk of any change in characters which have hitherto generally been considered sufficiently stable for use in taxonomy.

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