HEMIN BIOSYNTHESIS IN *HAEMOPHILUS*

DAVID C. WHITE[1] AND S. GRANICK

The Rockefeller Institute, New York, New York

Received for publication 19 November 1962

ABSTRACT

WHITE, DAVID C. (The Rockefeller Institute, New York, N.Y.) AND S. GRANICK. Hemin biosynthesis in *Haemophilus*. J. Bacteriol. **85**:842–850. 1963.—Hemin-independent *Haemophilus* species have been shown to form hemin by the classical hemin biosynthetic pathway. Three distinct species of *Haemophilus* [*H. influenzae*, *H. aegyptius*, and *H. canis* (*H. haemoglobinophilus*)] all lost the enzymatic capacities to convert δ-aminolevulinic acid to protoporphyrin, which accounts for their dependence on hemin for growth. The strain of *H. aegyptus* tested cannot form hemin from protoporphyrin, can be transformed with deoxyribonucleic acid (DNA) from *H. influenzae*, and the resultant progeny have the enzymatic activity to convert protoporphyrin to hemin. Attempts to transform these species to hemin independence with DNA from hemin-independent *H. parainfluenzae* are unsuccessful under conditions where streptomycin resistance is readily transformed.

The hemin biosynthetic pathway is peculiar in that in almost every detail the same enzymatic steps are used in animals, plants, and microorganisms (see review by Granick and Mauzerall, 1961). The steps in the formation of protoporphyrin (PROTO) are the same whether the end product is cytochrome-hemin, hemoglobin-hemin, or chlorophyll.

The first step in this biosynthesis consists of the formation of δ-aminolevulinic acid (ALA). This amino ketone is formed from either α-ketoglutarate or succinate and glycine at a branch point in the tricarboxylic acid cycle. Two linear ALA molecules are condensed to form into the five-membered pyrrole ring, porphobilinogen (PBG). Four of these PBG molecules then condense to form uroporphrinogen III (isomer III), the reduced hexahydroporphyrin with side chains of four acetic and four propionic acids. The uroporphrinogen acetic acid side chains are then successively decarboxylated to coproporphyrinogen. Two of the propionic acid groups are oxidatively decarboxylated to form protoporphrinogen. Protoporphyrinogen is then oxidized to PROTO, and iron is chelated to form hemin. The hemin thus formed is modified into the various cytochrome hemins, used directly in hemoglobin or catalase, or formed into chlorophyll.

Naturally occurring hemin-requiring species are rare and confined to microorganisms (Lascelles, 1961). The most extensively studied of the hemin-requiring microorganisms is *Haemophilus influenzae*. The examination of the missing enzymatic steps in this biosynthetic pathway is the subject of this paper.

Davis (1917) reported that the heat-stable hemoglobin requirement for growth of *H. influenzae* could be replaced with hemin. Lwoff and Lwoff (1937) showed that this species grown in limiting hemin had both an immediate increase in respiratory activity and a final level of growth that was directly proportional to the amount of hemin added to the medium. Granick and Gilder (1946) and Gilder and Granick (1947) showed that *H. influenzae* could utilize mesohemin, deuterohemin, and hematohemin as well as hemin, but that the vinyl groups had to be present on the porphyrin ring for the bacteria to be able to use the porphyrin itself as the growth factor. They showed that aerobic and anaerobic growth is dependent on hemin in proteose peptone medium. Brumfitt (1959) used porphyrin intermediates as growth-stimulating substances on agar surfaces containing inhibitory peroxides. ALA, PBG, uroporphyrin (URO), or coproporphyrin (COPRO) did not stimulate the growth under these conditions.

MATERIALS AND METHODS

Solutions of hemins and porphyrins were prepared as described by Granick and Gilder (1946). URO and PBG were synthesized by and

[1] Present address: Department of Biochemistry, University of Kentucky College of Medicine, Lexington

provided through the generosity of S. F. McDonald. COPRO III was isolated from diphtheria toxin broth supplied by F. H. Clarke of Lederle Laboratories. Coproporphyrinogen was prepared from COPRO, using methods described by Mauzerall and Granick (1958) and Sano and Granick (1960). ALA, aminoacetone, and PBG were assayed by the methods of Mauzerall and Granick (1956). Aminoacetone, PBG, and ALA were separated chromatographically by the methods of Elliot (1960) and Urata and Granick (*unpublished data*).

Bacterial fractions were prepared either with alumina, as described by White and Smith (1962), or by shattering at the temperature of liquid nitrogen (Moses, 1955).

Porphyrin synthetic capacities of bacteria and bacterial fractions were measured by incubation in 13 ml of 0.05 M phosphate buffer (pH 7.0) in 25-ml Erlenmeyer flasks shaken in the dark at 37 C. At zero and various times thereafter 2-ml samples were withdrawn and added to 2 ml of 0.3 M trichloroacetic acid. After 10 min at room temperature, the mixture was centrifuged at 8,000 × *g* for 10 min. Part of the supernatant liquid was used directly to measure PBG, and part was chromatographed to separate aminoacetone and ALA and then assayed. The porphyrins were measured after extracting the incubation mixture with 2 N HCl in a dim light for 30 min to allow auto-oxidation to the porphyrin (Sano and Granick, 1960). After centrifugation, the absorption maximum was measured spectrophotometrically and calculated as COPRO [$\epsilon_{mM}^{402\ m\mu} = 470$, 1 N HCl].

The porphyrins were identified chromatographically by extracting the incubation solution (made to pH 3.5 with acetic acid) with cyclohexanone. The organic phase was then washed with water, concentrated by flash evaporation in the presence of ethylenediaminetetraacetic acid, and separated by ascending chromatography using 2.6 M lutidine and 0.7 M NH_4OH (10:7; v/v) as solvent. The porphyrins separate by carboxyl group number (Nicholas and Rimington, 1951). The isomers of COPRO were separated using the system developed by Mauzerall (1960).

Bacterial growth was measured by the turbidity with the Klett colorimeter using the band pass filter (580 to 600 mμ) in 13-mm test tubes. The concentration of intermediates used were: ALA, 3×10^{-3} M; PBG, 5×10^{-5} M; COPRO, 2×10^{-5} M, and hemin, 6×10^{-6} M. ALA was sterilized by filtration and added from a stock solution in 0.05 M acetate buffer (pH 4.5). PBG was dissolved in 0.1 M KOH and added to the medium. COPRO, PROTO, and hemin were added from stock solutions made up of 50% ethanol and 0.01 M KOH. The inoculum consisted of about 10^5 late stationary-phase cells incubated for 4 hr without hemin at 37 C. Growth was measured after 36 hr at 37 C in 50-ml Erlenmeyer flasks containing 10 ml of medium. The pH at the beginning and the end of the experiment was 7.6. For growth in coproporphyrinogen, flasks fitted with fritted-glass dispersion discs and ⧩ joints were thoroughly deoxygenated with hot copper scrubbed nitrogen after inoculation, and the very alkaline solution of coproporphyrinogen formed during the reduction of COPRO was added rapidly. The buffer capacity of the medium neutralized the added alkali, and the flasks were incubated in the dark for 48 hr at 37 C. Doubling time was measured from the linear portion of the growth curve in 125-ml side-arm Erlenmeyer flasks incubated at 37 C without shaking.

Bacterial strains used in this study were: *H. influenzae* rd Garf, *H. influenzae* rd Sano, *H. aegyptus* 15, and *H. parainfluenzae* Boss (supplied by G. Leidy of Columbia University); *H. suis* 3812, *H. suis* 3090, and *H. suis* 7356-8 (isolated by R. E. Shope, Rockefeller Institute); *H. parainfluenzae* J-66 and *H. parainfluenzae* K-17 (isolated by E. L. Biberstein, University of California); *H. haemolyticus* 10014, *H. parainfluenzae* 9796, and *H. piscium* 10801 (American Type Culture Collection); *H. canis* 1659 (*H. haemoglobinophilus*) and *H. aphrophilus* 5886 (National Collection of Type Cultures, London). A proteose peptone base medium described by White (1963), in which growth of hemin-requiring *Haemophilus* is dependent on added hemin, was used in this study.

The transformation techniques and deoxyribonucleate preparations were those described by Leidy, Jaffee, and Alexander (1962). Difference spectra were measured as reported previously (White, 1962). Protein was measured by the biuret method (White, 1962).

Results

Intermediates of hemin biosynthesis as growth factors. In proteose peptone medium, the final growth achieved and the generation time of hemin-requiring *Haemophilus* species were pro-

TABLE 1. *Intermediates of hemin biosynthesis as growth factors for Haemophilus species*

Supplement to medium	Turbidity (Klett units)			
	Hemin requiring			Hemin independent
	H. influenzae	*H. aegyptus*	*H. canis*	*H. parainfluenzae*
None	0	0	0	215
Hemin	110	115	98	197
ALA	0	0	0	210
ALA + hemin	112	100	92	190
PBG	0	0	0	220
PBG + hemin	105	120	99	198
COPRO	0	0	0	215
COPRO + hemin	110	117	100	200
PROTO	105	0	100	170
PROTO + hemin	110	110	98	190

portional to the concentration of hemin added to the medium (*unpublished observation*). Using this medium, the various intermediates of hemin biosynthesis can be tested for growth-factor activity (Table 1). *H. parainfluenzae*, *H. piscium*, *H. aphrophilus*, *H. suis*, *H. parainfluenzae* J-66, and *H. parainfluenzae* K-17 grew in medium containing less than 10^{-9} M hemin or 10^{-8} M ALA. All these species can form a cytochrome system as detected by a typical oxygen uptake with reduced diphosphopyridine nucleotide (DPNH) or by difference spectra (White, 1963) and presumably have the complete hemin biosynthetic system. Three species, *H. influenzae*, *H. aegyptus*, and *H. canis*, were unable to grow in proteose peptone medium unless hemin was present. *H. influenzae* and *H. canis* were able to grow with PROTO in this medium. The intermediates ALA, PBG, and COPRO were neither inhibitory nor stimulatory to growth (Table 1). The fully reduced hexahydroporphyrins rather than the porphyrins, themselves, are the actual intermediates in this biosynthetic pathway. If the porphyrins were reduced and added to an inoculated thoroughly deoxygenated culture apparatus and incubated in the dark, a reproducible increase in turbidity always resulted (Table 2). This increase consisted of between three and five cell divisions after which growth

TABLE 2. *Anaerobic growth of Haemophilus with coproporphyrinogen*

Bacteria	Turbidity (Klett units)			
	Control	Control + 2×10^{-6} M coproporphyrinogen	Control + 2×10^{-6} M hemin	Control + 2×10^{-6} M coproporphyrinogen and 2×10^{-6} M hemin
Hemin-requiring species				
H. influenzae	0	13	35	75
H. aegyptus	0	15	32	57
H. canis	0	19	42	66
Hemin-independent species				
H. parainfluenzae	110	120	108	—

stopped. This growth was dependent on added coproporphrinogen. The porphyrin remained reduced throughout the growth period as indicated by the development of a characteristic pink color upon opening the vessel at the end of the experiment. No new hemin formation could be detected after growth in coproporphyrinogen.

The hemin-requiring species could store sufficient hemin to undergo five divisions if grown in a medium where hemin was not growth-limiting. Incubation of a large inoculum in hemin-free growth resulted in an increase in cell density that stops after 3 to 4 hr. If hemin was added there was an immediate resumption of growth.

Bacteria excrete porphyrins into the medium during growth (Lascelles, 1961); this is primarily COPRO III. The hemin-independent species produce between 10 and 30 μmoles COPRO per liter during a growth cycle, while the hemin-requiring species form no detectable porphyrin during growth. The porphyrin was extracted by acetic acid-ethylacetate, recovered, and assayed spectrophotometrically in 1 M HCl. No URO could be detected by extraction with cyclohexanone.

Enzymatic activities in hemin biosynthesis. The initial step in the formation of hemin is the production of ALA from succinyl-coenzyme A (CoA), pyridoxal phosphate, and glycine. The enzyme systems responsible for these reactions

have not thus far been demonstrable in *Haemophilus* preparations. The incubation mixtures used successfully by Lascelles (1959, 1960) and Kikuchi et al. (1958) for photosynthetic bacterial preparations and Granick and Urata (1962) for mitochondrial preparations were tried with various whole- and broken-cell preparations of hemin-independent *Haemophilus* species without success.

Another amino ketone, aminoacetone, appeared in the medium after growth, at concentrations of 10 to 30 μM per liter of medium for both hemin-requiring and hemin-independent species. It can be formed from threonine as discovered by Elliott (1960) or from acetyl-CoA, pyridoxal phosphate, and glycine by an enzyme with similar activity to the ALA-forming system.

If cell-free preparations of four hemin-independent *Haemophilus* species were incubated with ALA, the intermediates to COPRO could be demonstrated. PBG and the porghyrinogens with eight, seven, six, five, and four free carboxyl groups could be detected. The ALA could be accounted for within 10% as PBG, porphyrin, or ALA (Table 3), using cell-free preparations made with alumina at concentrations between 100 and 200 mg of bacterial protein. If the porphyrinogens were oxidized in dim light and air and extracted, lutidine-ammonia chromatography separated the intermediates by the number of free carboxyl groups on the porphyrin

TABLE 3. *Formation of porphyrin intermediates from ALA by hemin-independent Haemophilus cell-free preparations after incubation**

Determination	*H. aphrophilis*	*H. haemolyticus*	*H. parainfluenzae*	*H. suis*
ALA added	984	354	1,080	610
PBG formed	41	29	64	14
Porphyrin formed	43	33	73	22
ALA recovered	690	17	520	357
ALA accounted for	1,116 (113%)	339 (96%)	1,230 (113%)	561 (93%)
ALA equivalents				
ALA unutilized (%)	70	5	48	59
PBG recovered (%)	8	16	11	5
Porphyrin formed (%)	35	75	54	29

* Incubation was for 6 hr. Results expressed as μmoles per 100 mg of bacterial protein.

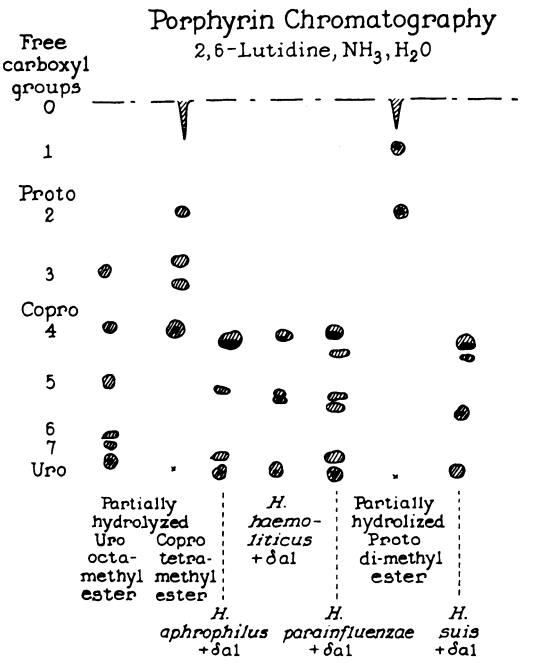

FIG. 1. *Porphyrin chromatogram of intermediates formed from δ-aminolevulinic acid by Haemophilus species. Porphyrins were identified by their red fluorescence.*

ring (Fig. 1). Partially hydrolyzed porphyrin methyl esters can be used as markers.

The enzymes responsible for coproporphyrinogen formation were found in the supernatant after centrifugation at 100,000 × *g* for 1 hr. Repeating the experiment above with the supernatant fraction of this centrifugation gave results nearly identical to those of Table 3.

The physiologically active isomer of COPRO is isomer III. The proportions of isomers formed with the cell-free preparations can be determined by careful descending chromatography (Fig. 2). The presence of both isomers I and III is evidence that *Haemophilus* forms uroporphyrinogen from PBG by the classical two-enzyme process (Bogorad, 1958*a*, *b*). Prolonged storage of preparations at −20 C increased the proportion of isomer I.

Using these methods, results with similar preparations of the hemin-requiring species are illustrated in Tables 4 and 5. Essentially no PBG or porphyrin was formed from ALA, and ALA could be recovered almost quantitatively. After incubation with PBG, about 10% of the porphyrin formed by a preparation of hemin-independent *H. parainfluenzae* was detected.

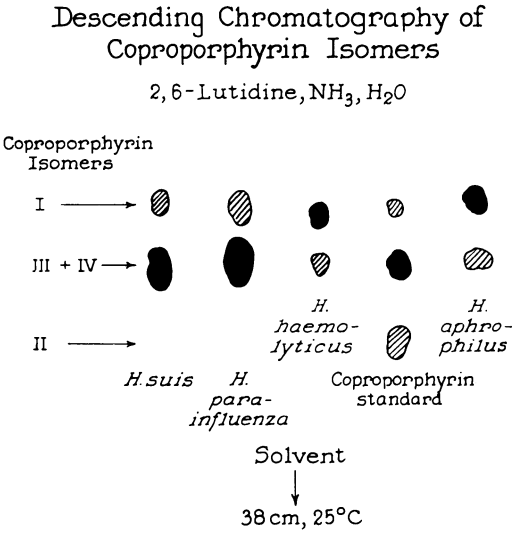

FIG. 2. *Coproporphyrin isomer chromatogram separated by descending chromatography in the system described in Materials and Methods.*

TABLE 4. *Formation of porphyrins from ALA by hemin-requiring Haemophilus cell-free preparations after incubation**

Determination	*H. influenzae*	*H. aegyptus*	*H. canis*
ALA added	109	4,740	392
ALA recovered	107 (97%)	4,600 (99%)	395 (101%)
PBG formed	<0.5	<0.5	<0.5
Porphyrin formed	<0.001	<0.001	<0.001

* Incubation was for 6 hr. Results expressed as μmoles per 100 mg of bacterial protein.

TABLE 5. *Formation of porphyrin intermediates from porphobilinogen (PBG) by hemin-requiring Haemophilus cell-free preparations after incubation**

Determination	*H. influenzae*	*H. aegyptus*	*H. canis*	*H. parainfluenzae*
PBG added	37	153	123	121
PBG recovered in 6 hr	25	115	108	70
Porphyrin formed	0.5	5.1	2.0	16.5
PBG accounted for	27 (73%)	135 (88%)	116 (95%)	136 (112%)

* Incubation was for 6 hr. Results expressed in μmoles per 100 mg of bacterial protein.

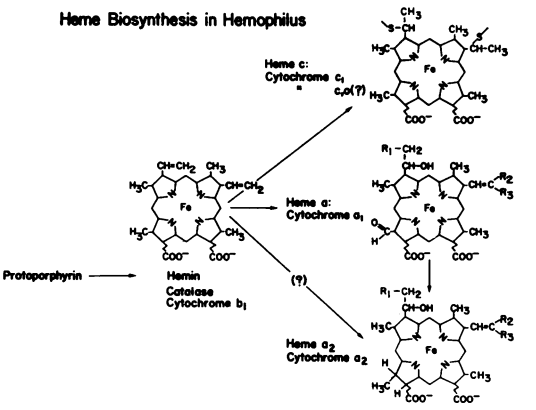

FIG. 3. *Hemin biosynthesis in the hemin-requiring Haemophilus. The structures of the cytochrome hemins formed by Haemophilus are given: hemin c (Paul, 1951), hemin a_1 (Lemberg, 1961), and hemin a_2 (Barrett, 1956).*

This porphyrin, formed during incubation with PBG in the hemin-requiring cell preparations, was URO (by chromatography) and was formed by the nonenzymatic condensation of PBG. Boiling the *H. influenzae* preparation had no effect on the amount of URO formed on incubation with PBG. The hemin-requiring species were unable to convert the uroporphyrinogen formed nonenzymatically (about 10 mμmoles) to coproporphyrinogen.

The activity of the enzyme which oxidatively decarboxylates two propionic acid side chains of coproporphyrinogen III to protoporphyrinogen IX could not be demonstrated, despite repeated attempts in either hemin-independent or hemin-requiring species. The enzyme that inserts iron into PROTO to convert it to heme was present in all *Haemophilus*, except for the one strain of *H. aegyptus* tested. All the *Haemophilus* species were able to convert hemin into the hemin prosthetic groups of the cytochromes (Fig. 3). These cytochromes were demonstrated in *H. canis* grown with PROTO in the difference spectra of Fig. 4. Here, bacteria whose electron-transport cytochrome system was reduced by DPNH were compared with a similar suspension of bacteria whose cytochrome system was oxidized.

Transformation of the hemin requirement. The *Haemophilus* group is characterized by the capacity of transformation of recipient populations with deoxyribonucleate isolated from other species (Leidy, Hahn, and Alexander, 1956,

1959; Schaeffer, 1956, 1958). Streptomycin resistance was readily transformed between species, but neither group was able to transform hemin independence. The hemin requirement is particularly interesting as it has never been shown to revert.

The finding that *H. aegyptus* could not grow on PROTO but required hemin suggested the absence of the enzyme that catalyzes the insertion of iron into the porphyrin ring. Using deoxyribonucleate isolated from *H. influenzae* (streptomycin-resistant, able to grow on protoporphyrin), transformation to the ability to grow on protoporphyrin occurred readily (Table 6). There was no evidence of linkage to streptomycin, and this

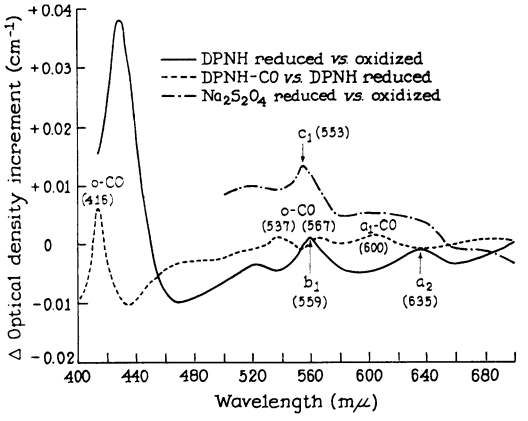

FIG. 4. *Difference spectra of Haemophilus canis grown in proteose peptone with protoporphyrin (0.5 μg/ml). Bacteria suspended in 0.05 M phosphate buffer (pH 7.0) with 20% (v/v) glycerin.*

transformation was completely inhibited by incubating the recipient population with crystalline deoxyribonuclease prior to the addition of transforming deoxyribonucleate. Addition of $FeCl_2$ (1.4×10^{-5} M) or thioglycolate (1.75×10^{-3} M) had no effect on the transformation or the deoxyribonuclease control. The resultant transformants were able to grow as rapidly and showed identical final growth in proteose peptone medium with limiting amounts of protoporphyrin as the naturally occurring PROTO-utilizing species of *H. influenzae* and *H. canis* (Fig. 5). Demonstration that the transformed activity was the enzyme in the transformant, *H. aegyptus* (p+), was suggested by the disappearance of 54.5% of added protoporphyrin incubated with whole cells as compared with 13% in an identical preparation of nontransformed *H. aegyptus* (p−). The incubation mixture contained: protoporphyrin, 0.02 μmoles; $FeSO_4$, 0.02 μmoles; thioglycolate, 40 μmoles; 100 μM tris buffer, and bacterial protein, 40 mg per ml; with the incubation in the dark for 3 hr at 37 C in nitrogen, using the technique of Labbe and Hubbard (1960).

Attempts to transform the hemin-requiring species to hemin independence were uniformly unsuccessful under conditions where transformation to streptomycin resistance readily occurred. If the selection for transformants is done in proteose peptone medium, five or six enzymatic activities must be functional for hemin-independent growth. Addition of ALA (10^{-4} M) or coproporphyrinogen (10^{-6} M) to the trans-

TABLE 6. *Interspecific transformations of Haemophilus**

Recipient species	Donor deoxyribonucleate	Transformants in 10^8 recipients		
Haemophilus aegyptus (Sm^s, P^-)	*H. influenzae* (Sm^r, P^+)	$Sm^s \rightarrow Sm^r$ 1×10^5	$P^- \rightarrow P^+$ 1.1×10^5	$Sm^sP^- \rightarrow Sm^rP^+$ 0
H. aegyptus (Sm^s, H^-)	*H. parainfluenzae* (Sm^r, H^+)	$Sm^s \rightarrow Sm^r$ 5.6×10^5	$H^- \rightarrow H^+$ 0	$Sm^sH^- \rightarrow Sm^rH^+$ 0
H. influenzae (Sm^s, H^-)	*H. parainfluenzae* (Sm^r, H^+)	$Sm^s \rightarrow Sm^r$ 1.7×10^5	$H^- \rightarrow H^+$ 0	$Sm^sH^- \rightarrow Sm^rH^+$ 0
H. parainfluenzae (Sm^s, DPN^-)	*H. canis* (Sm^r, DPN^+)	$Sm^s \rightarrow Sm^r$ 2.7×10^2	$DPN^- \rightarrow DPN^+$ 0	
H. parainfluenzae (Sm^s, DPN^-)	*H. aphrophilus* (Sm^r, DPN^+)	$Sm^s \rightarrow Sm^r$ 1.2×10^4	$DPN^- \rightarrow DPN^+$ 0	

* Markers are: Sm^r, streptomycin resistance; Sm^s, streptomycin sensitivity; P^+, growth on PROTO; P^-, no growth with PROTO; H^+, growth with hemin; H^-, hemin requiring; DPN^+, DPN independent; DPN^-, DPN requiring.

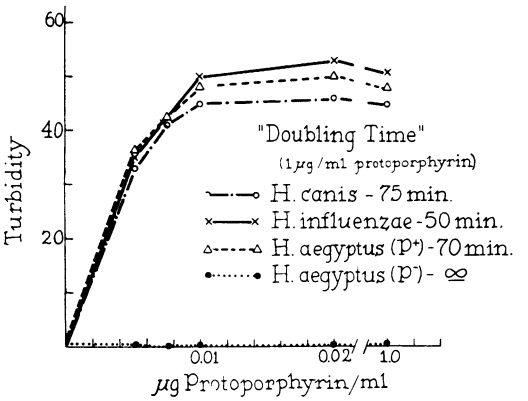

FIG. 5. *Variation in final growth level in proteose peptone medium with protoporphyrin concentration.*

forming medium would necessitate the activities of four or five enzymes for growth in ALA and one enzyme activity for growth in coproporphyrinogen, if the bacteria are permeable to these substrates. Adding these substrates had no effect on the transformation. For coproporphyrinogen, incubation must be anaerobic and in the dark. If sufficient hemin to allow two divisions was added in the presence of these substrates after exposure to deoxyribonucleate, the minimal growth was the same after transformation with deoxyribonucleate from hemin-independent species, transformation with deoxyribonucleate from hemin-requiring species, and transformation with the deoxyribonuclease-treated controls, indicating no transformation to hemin independence (Table 6).

If spheroplasts were made of *H. parainfluenzae* by growth in 3 M sucrose and 50 units of penicillin and then lysed, the lysate was capable of transforming streptomycin resistance to the streptomycin-sensitive recipient *H. influenzae*. There are no deoxyribonuclease activity and no viable cells in this lysate. Presumably, this spheroplast lysate should represent the least-manipulated deoxyribonucleate-ribonucleate preparation available. Using this preparation, no transformation of *H. influenzae* to hemin independence occurred.

The *Haemophilus* species have another requirement for pyridine nucleotide, which is the same in all species tested (Shifrine and Biberstein, 1960). All these species are able to grow on nicotinamide riboside but not nicotinic acid or nicotinamide. Again deoxyribonucleate from species not requiring diphosphopyridine nucleotide (DPN) was not able to transform to DPN independence under conditions where streptomycin resistance went readily (Table 6). In these experiments, selection for DPN independence was made directly, in the presence of nicotinic acid (10^{-3} M), or after a generation in DPN and treatment with *Neurospora* diphosphopyridine nucleotidase.

DISCUSSION

Of those strains of the *Haemophilus* species studied, the hemin-independent group is comprised of: *H. parainfluenzae*, *H. aphrophilus*, *H. suis*, *H. piscium*, and the animal *H. parainfluenzae* J-66 and K-17. All these species will grow in proteose peptone medium, which contains no detectable hemin, and during growth form a cytochrome system containing four types of hemin. The enzyme that converts ALA to PBG, the enzymes converting PBG to uroporphyrinogen III, and the enzyme that forms coproporphyrinogen III from uroporphyrinogen III have been demonstrated to be present. *H. haemolyticus* is believed to be part of this group, as it has the enzymatic activities found in the others, but as yet has not been grown in simplified medium free of hemin. For *H. haemolyticus* to grow, no hemin need be added to Brain Heart Infusion Broth. If small amounts of hemin are required, much less is needed for growth to comparable density than in the three species described below. The evidence that *H. haemolyticus* can form ALA is the finding of coproporphyrinogen and COPRO in the medium after growth. The conversion of coproporphyrinogen to PROTO has not been demonstrated.

The hemin-requiring species must have hemin present to grow in proteose peptone medium. The final density of growth is proportional to the hemin concentration up to 10^{-7} M for the three species, *H. influenzae*, *H. aegyptus*, and *H. canis*. All three lost the enzymatic capacity to form PBG from ALA, uroporphyrinogen III from PBG, coproporphyrinogen III from uroporphyrinogen III, and could not grow on coproporphyrinogen III. *H. aegyptus* cannot form hemin from PROTO, but the others can form the cytochrome hemins from PROTO. It has not been possible to detect the formation of ALA by these three hemin-requiring species.

The advantage of the *Haemophilus* system is the availability of transformation with isolated deoxyribonucleate between species. In the case

of the protoporphyrin-iron inserting enzyme, transformation presumably has shown this hemin biosynthetic enzyme to be inherited like other enzyme systems. The nontransformability to hemin or deoxyribonucleate independence probably represents the fact that a number of genes must be transformed in a single bacterium to provide a clone of hemin- or deoxyribonucleate-independent progeny. In the course of many generations in the presence of the required factor, secondary mutations that are not now of selective disadvantage may have occurred, which makes the possibility of a recombination to hemin or deoxyribonucleate independence even less likely. To test whether the genes for the missing enzymes are present but inactive requires the isolation of one-enzyme mutants. These auxotrophs have thus far not been isolated.

ACKNOWLEDGMENTS

The authors are indebted to G. Leidy, who generously provided us with her latest methods of transformation and growth and transformable strains of *Haemophilus*. We wish to thank R. E. Shope and E. L. Biberstein for cultures, S. F. McDonald for porphobilinogen and uroporphyrin, F. H. Clarke for diphtheria toxin broth, and D. Mauzerall and G. Urata for many stimulating discussions.

LITERATURE CITED

BARRETT, J. 1956. The prosthetic group of cytochrome a_2. Biochem. J. **64**:626–639.

BOGORAD, L. 1958*a*. The enzymatic synthesis of porphyrins from porphobilinogen I, uroporphyrin I. J. Biol. Chem. **233**:501–509.

BOGORAD, L. 1958*b*. The enzymatic synthesis of porphyrins from prophobilinogen II, uroporphyrin III. J. Biol. Chem. **233**:510–515.

BRUMFITT, W. 1959. Some growth requirements of *Haemophilus pertussis*. J. Pathol. Bacteriol. **77**:95–100.

DAVIS, J. D. 1917. Food accessory factors (vitamines) in bacterial culture with especial reference to *Hemophilus bacillus influenzae*. J. Infect. Diseases **21**:392–402.

ELLIOTT, W. H. 1960. Aminoacetone formation by *Staphylococcus aureus*. Biochem. J. **74**: 478–485.

GILDER, H., AND S. GRANICK. 1947. Studies on the *Hemophilus* group of organisms. Quantative aspects of growth on various prophyrin compounds. J. Gen. Physiol. **31**:103–117.

GRANICK, S., AND H. GILDER. 1946. The porphyrin requirements of *Haemophilus influenzae* and some functions of the vinyl and propionic acid side chains of heme. J. Gen. Physiol. **30**:1–13.

GRANICK, S., AND D. MAUZERALL. 1961. The metabolism of heme and chlorophyll, p. 525–625. *In* D. M. Greenberg [ed.], Metabolic pathways, vol. 3. Academic Press, Inc., New York.

GRANICK, S., AND G. URATA. 1962. Specificities of α-aminoketones and the induction of δ aminolevulinic acid (ALA) synthesis in liver mitochondria. Federation Proc. **21**:156.

KIKUCHI, G., A. KUMAR, P. TALMAGE, AND D. SHEMIN. 1958. The enzymatic synthesis of δ-aminolevulinic acid. J. Biol. Chem. **233**: 1214–1219.

LABBE, R. F., AND N. HUBBARD. 1960. Preparation and properties of the iron-protoporphyrin chelating enzyme. Biochim. Biophys. Acta **41**:185–191.

LASCELLES, J. 1959. Adaptation to form bacteriochlorophyll in *Rhodopseudomonas spheroides*: changes in activity of the enzymes concerned in pyrrole synthesis. Biochem. J. **72**:508–518.

LASCELLES, J. 1960. The synthesis of enzymes concerned in bacteriochlorophyll formation in growing cultures of *Rhodopseudomonas spheroides*. J. Gen. Microbiol. **23**:487–498.

LASCELLES, J. 1961. Synthesis of tetrapyrroles by microorganisms. Physiol. Rev. **41**:417–441.

LEIDY, G., E. HAHN, AND H. E. ALEXANDER. 1956. On the specificity of the desoxyribonucleic acid which induces streptomycin resistance in *Hemophilus*. J. Exptl. Med. **104**:305–320.

LEIDY, G. E., E. HAHN, AND H. E. ALEXANDER. 1959. Interspecific transformation of *Hemophilus*. A possible index of relationship between *H. influenzae* and *H. aegyptus*. Proc. Soc. Exptl. Biol. Med. **102**:86–88.

LEIDY, G., I. JAFFE, AND H. E. ALEXANDER. 1962. Emergence of competence (for transformation) of three *Hemophilus* species in a chemically defined environment. Proc. Soc. Exptl. Biol. Med. **111**:725–731.

LEMBERG, R. 1961. Cytochromes of group a and their prosthetic groups. Advan. Enzymol. **23**:265–321.

LWOFF, A., AND M. LWOFF. 1937. Role physiologique de l'hemine pour *Hemophilus influenza, Pfeiffer*. Ann. Inst. Pasteur **59**:129–136.

MAUZERALL, D. 1960. The thermodynamic stability of the porphyrinogens. J. Am. Chem. Soc. **82**:2601–2605.

MAUZERALL, D., AND S. GRANICK. 1956. The occurrence and determination of δ-aminolevulinic acid and porphobilinogen in urin. J. Biol. Chem. **219**:435–446.

MAUZERALL, D., AND S. GRANICK. 1958. Porphyrin synthesis in erythrocytes. III. Uroporphyrin

and its decarboxylase. J. Biol. Chem. **232**: 1141–1162.

MOSES, V. 1955. Tricarboxylic acid cycle reactions in the fungus *Zygorrhynchus moelleri*. J. Gen. Microbiol. **13**:235–251.

NICHOLAS, R. E. H., AND C. RIMINGTON. 1951. Paper chromatography of porphyrins. Some hitherto unrecognized porphyrins and further notes on the method. Biochem. J. **48**:306–309.

PAUL, K. G. 1951. The porphyrin component of cytochrome c and its linkage to the protein. Acta Chem. Scand. **5**:389–405.

SANO, S., AND S. GRANICK. 1960. Mitochondrial coproporphyrinogen oxidase and protoporphyrin formation. J. Biol. Chem. **236**:1173–1180.

SCHAEFFER, P. 1956. Transformation interspécifique chez des bactéries du genre *Hemophilus*. Ann. Inst. Pasteur **91**:192–211.

SCHAEFFER, P. 1958. Interspecific reactions in bacterial transformations. Symp. Soc. Exptl. Biol. **12**:60–74.

SHIFRINE, M., AND E. L. BIBERSTEIN. 1960. A growth factor for *Haemophilus* species secreted by a pseudomonad. Nature **187**:623.

WHITE, D. C., AND L. SMITH. 1962. Hematin enzymes of *Hemophilus parainfluenzae* J. Biol. Chem. **237**:1332–1336.

WHITE, D. C. 1962. Cytochrome and catalase patterns during the growth of *Haemophilus parainfluenzae*. J. Bacteriol. **83**:851–859.

WHITE, D. C. 1963. Respiratory systems in hemin-requiring *Haemophilus* species. J. Bacteriol. **85**:84–96.