ACTION OF *HAEMOPHILUS* CULTURES ON δ-AMINOLEVULINIC ACID

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ABSTRACT

BIBERSTEIN, ERNST L. (University of California, Davis), PATRICIA D. MINI, AND MARJORIE G. GILLS. Action of *Haemophilus* cultures on δ-aminolevulinic acid. J. Bacteriol. **86**:814–819. 1963.—Utilization of δ-aminolevulinic acid (ALA) by strains of *Haemophilus* recovered from various sources was investigated. With the 37 cultures studied, there was perfect correlation between absence of hemin requirement and ability to convert ALA to porphyrin. A total of 29 strains, including representatives of *H. parainfluenzae*, *H. parahaemolyticus*, *H. "parasuis,"* and *H. gallinarum*, fell into this group. The remaining eight isolates, which were incapable of porphyrin synthesis from ALA, were strains of *H. influenzae*, *H. suis*, and a *Haemophilus* culture of uncertain classification obtained from a splenic abscess in a deer. In the active preparations, the products of synthesis included a mixture of porphyrins, porphobilinogen (PBG), and a pigment which absorbed light strongly at 470 to 480 mμ. Paper chromatographic studies of fractions of supernatants and cells revealed the presence of porphyrins having R_F values similar to those of uro- and protoporphyrins, as well as some intermediate rates of migration probably representing coproporphyrins. Porphyrins were found both intra- and extracellularly, and PBG and the pigment absorbing at 470 to 480 mμ were confined to the supernatant.

The classification of a gram-negative bacterium as *Haemophilus* rests upon demonstration of certain biosynthetic inadequacies concerning particularly the pyridine nucleotide coenzymes and iron porphyrin. These have been traditionally established by attempts to grow the organism on media lacking, or providing, one or both of the two types of growth factor. This method has two drawbacks. One is that the inoculum may contain sufficient amounts of the critical factor to permit growth for a variable number of passages. Another is the possible presence of traces of the growth substances in the test medium, too small to be detected by chemical or optical means but sufficient to satisfy growth-factor requirements. These drawbacks apply particularly to tests for the heme requirement of *Haemophilus*, whose needs for this substance are minute (0.01 to 0.05 μg/ml) and subject to further reduction under anaerobic conditions (Gilder and Granick, 1947). Moreover, unlike the pyridine nucleotide coenzymes, porphyrins withstand the rigors of media preparation and storage well. Therefore, since a synthetic medium capable of supporting growth of a representative spectrum of *Haemophilus* strains does not exist, cultural examination for prophyrin requirements of unknown strains can involve time-consuming serial transfers and even then leave the issue somewhat in doubt.

With the elucidation of porphyrin biosynthesis in higher organisms and bacteria, methods and reagents have become available for determining the porphyrin requirements without resort to growth studies. It was suggested (White, *personal communication*) that we test the ability of unknown cultures to convert δ-aminolevulinic acid (ALA) to porphyrins. The present paper reports the results of this attempt to determine the porphyrin needs of a variety of *Haemophilus* strains by testing their ability to synthesize porphyrins from ALA.

MATERIALS AND METHODS

Brief characterizations of the 37 strains examined are given in Table 1.

The organisms were propagated for 18 to 24 hr in Brain Heart Infusion broth (Difco) supplemented with the accessory factors required for maximal growth. All strains, except N51, were cultured in the presence of 1 μg each of nicotinamide adenine dinucleotide (NAD^+; Calbiochem) and hemin (Calbiochem) per ml of

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TABLE 1. *Ability of Haemophilus strains to convert ALA to porphyrin*

Strain designation	Source	ALA → P*	Suggested classification
b	H. E. Alexander, New York, N.Y.	−	*H. influenzae*
c	H. E. Alexander, New York, N.Y.	−	*H. influenzae*
d	H. E. Alxeander, New York, N.Y.	−	*H. influenzae*
641b	Margaret Pittman, Bethesda, Md.	−	*H. influenzae*
525	Margaret Pittman, Bethesda, Md.	−	*H. influenzae*
L54	Meningitis, man, Sacramento, Calif.	−	*H. influenzae*
7901	American Type Culture Collection, Washington, D.C.	+	*H. parainfluenzae*
9796	American Type Culture Collection, Washington, D.C.	+	*H. parainfluenzae*
J66	Nasopharynx, guanaco, San Diego, Calif.	+	*H. parainfluenzae*
0169	Upper respiratory passages, chicken, Davis, Calif.	+	*H. parainfluenzae*
0276	Upper respiratory passages, chicken, Davis, Calif.	+	*H. parainfluenzae*
0207	Upper respiratory passages, chicken, Davis, Calif.	+	*H. parainfluenzae*
14385	Upper respiratory passages, chicken, Davis, Calif.	+	*H. parainfluenzae*
H37	Avian coryza, chicken, Davis, Calif.	+	*H. gallinarum*
0083	Avian coryza, chicken, Davis, Calif.	+	*H. gallinarum*
0233	Avian coryza, chicken, Davis, Calif.	+	*H. gallinarum*
0222	Avian coryza, chicken, Davis, Calif.	+	*H. gallinarum*
M59	Pneumonia, pig, Neb.	+	*H. parasuis*
M76	Polyserositis, pig, Iowa	+	*H. parasuis*
M77	Polyserositis, pig, Iowa	+	*H. parasuis*
M78	Polyserositis, pig, Iowa	+	*H. parasuis*
M81	Septicemia, pig, Nev.	+	*H. parasuis*
M94	Nasopharynx, pig, Calif.	+	*H. parasuis*
M96	Nasopharynx, pig, Calif.	+	*H. parasuis*
M92	Nasopharynx, pig, Calif.	+	*H. parasuis*
M95	Nasopharynx, pig, Calif.	+	*H. parasuis*
M97	Nasopharynx, pig, Calif.	−	*H. suis*
K8	Septicemia, pig, Calif.	+	*H. parahaemolyticus*
K17	Arthritis, lamb, Calif.	+	*H. parahaemolyticus*
K45	Cerebral abscess, steer, Calif.	+	*H. parahaemolyticus*
K98	Polyserositis, pig, Calif.	+	*H. parahaemolyticus*
L20	Pneumonia, pig, Calif.	+	*H. parahaemolyticus*
L31	Pneumonia, pig, Calif.	+	*H. parahaemolyticus*
M57	Pneumonia, pig, Argentina	+	*H. parahaemolyticus*
M62	Pneumonia, pig, Calif.	+	*H. parahaemolyticus*
M68	Pneumonia, pig, Calif.	+	*H. parahaemolyticus*
N51	Abscess, deer, Calif.	−	*Haemophilus* sp.

* Ability to convert ALA to porphyrins.

medium. In the case of *H. gallinarum*, reduced NAD^+ (NADH, Calbiochem) was substituted for NAD^+, and 1% chicken serum was included (Page, 1961). Horse serum (1%) was added to all cultures of *H. suis* and *H. "parasuis."* Strain N51 did not grow with NAD^+ or NADH supplementation, but it could be propagated when 20% of the medium consisted of a filtrate from a broth culture of the Emperor penguin pseudomonad (Shifrine and Biberstein, 1960).

The method of determining conversion of ALA to porphyrin was essentially that described by Lascelles (1956). Washed bacterial cells containing 400 μg of protein nitrogen were suspended in 5 ml of sterile 0.1 M phosphate-0.0008 M magnesium sulfate buffer at pH 6.9. To this suspen-

sion were added 1.8 mg of ALA hydrochloride (Calbiochem). The mixture, along with a control suspension containing no ALA, was incubated on a shaker for about 15 hr at 37 C. At the end of this period, the tubes were examined for fluorescence under a Mineralight (3800 A; Ultraviolet Products, Inc., South Pasadena, Calif.). Spectrophotometric scanning was done on a Beckman DB spectrophotometer, with a recording attachment, against suspending fluid references.

Separation of porphyrins was attempted by ethyl acetate extraction of the cell-free supernatant, followed by extraction of the ethyl acetate phase with 1.5 N HCl. Paper chromatography followed Eriksen's (1953) procedure with a solvent system of 2,6-lutidine and water (5:3) in an atmosphere of 25% ammonia. Porphobilinogen (PBG) was determined qualitatively with Ehrlich's reagent.

RESULTS

Table 1 shows the results of incubating ALA with *Haemophilus* cells under the conditions described. Parallel cultural studies in which serial passage was attempted on media containing hemin only, NAD^+ only, and both hemin and NAD^+ revealed that all strains not requiring hemin as a nutrient were able to synthesize porphyrin from ALA, and those incapable of such synthesis required hemin. A strain unable to convert ALA to porphyrin, yet independent from hemin supplement, has not been found to date.

Red fluorescence was present in both cells and supernatants of reaction mixtures containing

TABLE 2. *R_F values of fluorescent substances obtained from Haemophilus cells and supernatants after incubation with ALA*

Determination	Strain			
	K45	M57	H37	K8
Ethyl acetate phase of supernatant extracted with HCl	0.80	0.98 0.18	0.91	0.91 0.95
Aqueous phase after ethyl acetate extraction	0.08	0.04	0.1 0.93	0.24
HCl cell digest	0.81	0.98 0.14	0.25	0.89 0.12

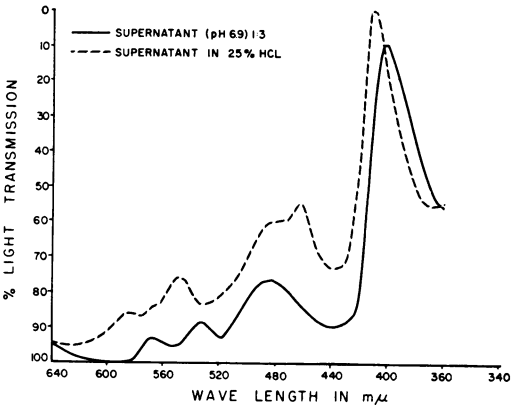

FIG. 1. *Light absorption by the supernatant of a suspension of Haemophilus parahaemolyticus in ALA after about 15 hr of incubation at 37 C.*

bacteria capable of porphyrin synthesis. When the supernatants were extracted with ethyl acetate, most of the fluorescence remained in the aqueous phase. Extraction of the ethyl acetate layer with 1.5 N HCl revealed fluorescence in this fraction as well. Chromatograms of the two fractions and of a 1.5 N HCl digest of the cells showed that a mixture of porphyrins with sharply differing migration rates was present. Some fluorescent spots migrated closely behind the solvent front as did a control consisting of protoporphyrin IX, and others remained near the origin, showing R_F values similar to uroporphyrin. Intermediate rates of migration, corresponding to porphyrins of varying degrees of decarboxylation, were also occasionally observed. The ethyl acetate phase contained predominantly fast-moving fluorescent substances, and most of the fluorescent material from the aqueous phase migrated slowly (Table 2).

Fluorescent supernatants were uniformly positive for PBG, and nonfluorescent ones were negative, as were all cells regardless of fluorescence. Spectrophotometric scanning of supernatants and cell digests revealed typical porphyrin spectra. The frequent asymmetry of absorption peaks again pointed to the presence of mixtures of porphyrins. Figure 1 shows a typical absorption spectrum of the crude supernatant of an *H. parahaemolyticus* K45 suspension at pH 6.9 and in 25% HCl. The peaks at 570, 535, and 405 mμ in the near-neutral system are consistent with known porphyrin bands. On occasion, an additional band was present at about 620 mμ, the location of band

I of porphyrin. Porphyrin band IV, located at about 500 mμ, was never observed in our preparations, presumably because it was overshadowed by the very prominent peak between 470 and 480 mμ, which does not fit any known porphyrin absorption band. Several other circumstances pointed to the likelihood that the substance responsible for this absorption band was distinct from the several porphyrins known to be present. In 25% HCl, all the identifiable porphyrin absorption peaks shifted in the infrared direction, while the peak at 470 to 480 mμ moved toward the ultraviolet and became diphasic. This phenomenon was observed with a number of strains (Fig. 1). In several tests, particularly when sonically disrupted cells (20 min in a 10-kc Raytheon sonic oscillator) were used in lieu of intact cells, absorption in the 470 to 480-mμ region was more intense than in the Soret area and elsewhere. Finally, only supernatants and their fractions showed light absorption in the region from 470 to 480 mμ. Cell extracts, although exhibiting the absorption properties of porphyrins, consistently lacked the band from 470 to 480 mμ (Fig. 2). The method of cell extraction, i.e., autoclaving for 60 min with 1.5 N HCl, was without adverse effect on absorption in this area when used on supernatants. On the contrary, the peak was substantially increased along with those peaks due to porphyrins, presumably owing to the conversion of PBG which disappeared in the process as measured by the Ehrlich reaction.

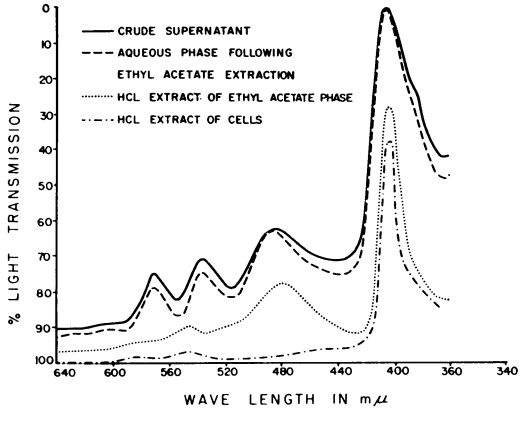

FIG. 2. *Light absorption by the supernatant, supernatant extracts, and cell extracts of a suspension of Haemophilus parahaemolyticus in ALA after 15 hr of incubation at 37 C. Note absence of absorption at 480 mμ by the cell extract.*

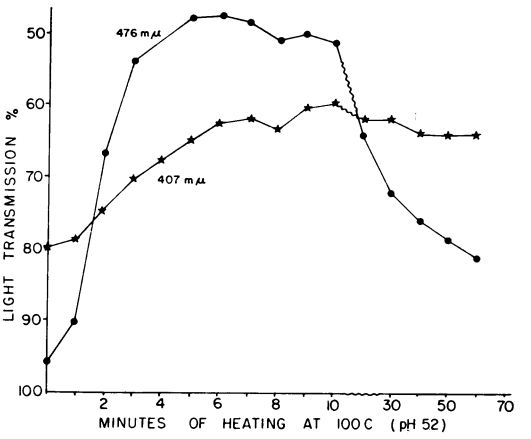

FIG. 3. *Effect of heating on the light absorption by the supernatant of Haemophilus parahaemolyticus suspension in ALA after incubation for 15 hr at 37 C. Prior to heating, the pH was brought to 5.2 with 2 M Na acetate-acetic acid buffer.*

The possibility was considered that the substance responsible for light absorption in the region from 470 to 480 mμ was related to, or identical with, the "yellow pigment" described by Brockman and Gray (1953). Further experiments bore out this assumption. When supernatants obtained by incubating ALA with *H. parahaemolyticus* K98 cells were brought to pH 5.2 by the addition of an equal volume of 2 M sodium acetate-acetic acid buffer and heated in a boiling-water bath, the peak in the critical region was increased substantially at first, although absorption due to porphyrins (as measured in the Soret band) increased considerably less. When heating was continued up to 60 min, the relative height of the two peaks approximated that prior to heating (Fig. 3). It was found that heating for 6 min produced maximal absorption in the critical area, with development of a distinct yellow color. When such a solution was permitted to stand at room temperature or in a refrigerator, a stringy precipitate was formed. This process could be hastened by repeated filtration through Whatman no. 1 filter paper. In successive filtrates, the height of the Soret peak relative to that at 470 to 480 mμ remained essentially constant. However, when the filter papers used in the procedures were washed lightly with 1.5 N HCl, the centrifuged washings exhibited only one absorption peak (i.e., at 473 mμ).

DISCUSSION

On the basis of the data presented, the ability of a *Haemophilus* culture to convert ALA to porphyrin is indicative of independence from hemin or other porphyrin growth factors. Conversely, inability to carry out this reaction implied, in the strains examined, an X-factor requirement. The possibility remains that types exist which neither synthesize porphyrins nor require them. Under present usage, such types would obviously qualify as *Haemophilus* only if they have a V-factor requirement.

All cultures of poultry origin proved to be able to utilize ALA in porphyrin synthesis. These included representatives of both the pathogenic, microaerophilic, and otherwise fastidious *H. gallinarum*, and the apparently nonpathogenic, less fastidious types, which are culturally indistinguishable from *H. parainfluenzae* and have therefore been included with them in our tabulation. The ability of *H. gallinarum* to synthesize porphyrin is in line with Page's (1961) finding that no X factor was required for the propagation of any strains of this organism. These observations do not agree with current species criteria as set forth in *Bergey's Manual* (Breed, Murray, and Smith, 1957).

Of the nonhemolytic cultures obtained from swine, only one failed to convert ALA and thus was the sole representative of the classical *H. suis* described by Lewis and Shüpe (1931). The other nine strains could reasonably be called *H. "parasuis,"* a term already introduced into the literature by White (1963). *H. "parasuis"* is readily differentiated from *H. parahaemolyticus* cultures of porcine origin by its failure to lyse bovine red blood cells, as well as by its more fastidious nature. The hemolytic isolates from swine will grow well on Proteose peptone agar with NAD^+, and *H. "parasuis"* requires serum or some other enrichment in addition.

The phenomena observed in connection with the synthesis of porphyrins from ALA differed in several respects from those previously recorded for bacteria. Lascelles (1956), working with *Rhodopseudomonas spheroides*, observed free porphyrins only in the supernatants of her reaction mixtures. In our tests, free porphyrin was invariably present in the cells as well. However, neither PBG nor the "yellow pigment" was ever encountered intracellularly. These findings might be interpreted as evidence of an extracellular site of porphyrin synthesis in our system, from which the porphyrins diffuse into the cells. Another, and perhaps more plausible, explanation is that all the conversion products originated in the cell and that PBG does not accumulate intracellularly but is promptly utilized for synthesizing porphyrins, thus allowing for no production of the yellow pigment. Although no attempt was made to identify chemically the porphyrins formed, the paper chromatograms point to a prevalence of uro- and protoporphyrin. Most other studies on bacterial porphyrin synthesis have revealed more substantial coproporphyrin components (Lascelles, 1962), as did White and Granick's (1963) recent investigation on *Haemophilus*.

The substance absorbing at 470 to 480 mμ resembles in most particulars the "yellow pigment" of Brockman and Gray (1953), who obtained it as a heating artifact from PBG solutions at pH 5.2 and suggested that it was "a 5:5 disubstituted dipyrryl methene or a bilene." We are not aware of its having been identified in bacterial or other biosynthesis of porphyrins. Recently, Bogorad (1962) described a uroporphyrinogen I precursor which, when oxidized, becomes an Ehrlich-negative, orange-red pigment absorbing visible light at about 500 mμ. Bogorad believed this substance also to be a dipyrrole. From the meager evidence, it would appear therefore that, in the process of porphyrin synthesis, monopyrrole intermediates, i.e., PBG and possibly other Ehrlich-positive substances, give rise to certain by-products that may turn out to be dipyrroles.

Since, in systems employing heme-requiring cells, no Ehrlich-reacting or light-absorbing substances were present, at least one gap in the pathway to heme synthesis can be pinpointed at the site of ALA conversion to PBG. Nothing is known regarding the ability of these strains to synthesize the immediate ALA precursor α-amino-β-keto-adipic acid. Apparently, the insertion of iron into the porphyrin ring lies within the capabilities of at least some of the heme-requiring strains, since Gilder and Granick (1947) and White and Granick (1963) found that protoporphyrin IX could serve as a hemin substitute for *H. influenzae*. The latter work demonstrated the inability of heme-requiring *Haemophilus* to utilize PBG, or any of the porphyrinogens on the direct pathway to hemin synthesis.

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