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THE EFFECT OF HAEMIN ON GROWTH AND CYTOCHROME PRODUCTION IN CORYNEBACTERIUM HAEMOLYTICUM

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In taxonomical studies on Corynebacterium haemolyticum the presence of cytochrome b_1 in the particulate fraction of microbial bodies was demonstrated (Patočka 1967, Mára et al. 1971). This finding distinguishes Corynebacterium haemolyticum from Streptococcus pyogenes group A. On the other hand cytochromes a and c which both are regularly produced by Corynebacterium diphtheriae along with cytochrome b, could not be found.

Patočka and Kalvodová (1969) observed a marked enhancement of L-forms formation after addition of hemin into the cultivation medium.

The present paper tries to determine the influence of haemin on the growth of Corynebacterium pyogenes at different cultivation conditions. On the basis of structural similarity the synthesis of cytochrome b and the concentration of haemin are put into relation.

MATERIAL AND METHODS

Bacteria and media. Corynebacterium haemolyticum strain K-501 and — in some experiments — strain K-29 were used. Bacterial growth was measured in two different fluid media: 1. Todd-Hewitt broth modified by the addition of 2 % of calf serum or 1 % glucose (TH). 2. Synthetic medium according to Wilson (1954) modified by the omission of agar and by the addition of 1 % human serum albumin instead of calf serum. Haemin (He or Ahe) was added as acethaemin in the following concentrations: 1, 5, 10 and 20 mcg/ml respectively. For the cytochrome assay Todd-Hewitt broth with 2 % calf serum (in some experiments 1 % glucose) (TH) was used. Acethemin was added in the following concentrations: 1, 5, 10 and

20 mcg/ml respectively. Two solid media were used: 1. Brain-Heart Infusion (Difco) with 5 % calf serum and 2 % agar added. 2. Tryptic Soy Broth (Difco) with 5 % calf serum and 2 % agar added (TSB). To both media acethaemin in concentrations given above was added.

Cultivation. For cytochrome assay bacteria were grown in static culture for 24—48 hours at 37 °C both at aerobic and anaerobic conditions (in N_2 atmosphere). In some experiments bacteria were grown in Erlenmeyer flasks with 10 % sheep blood agar, overlaied with broth, and shaken in a waterbath for 24 hours at 37 °C. For inoculation 10 ml of 24 hours broth culture were added into 500 ml of the medium.

Bacterial growth was measured in thin-walled test tubes filled with 5 ml of medium inoculated and shaken at 37 °C, or in test tubes sealed to the bottom of Erlenmeyer flasks. The volume of cultivation medium was in this case 20 ml.

Inoculum. Three times washed suspension of bacteria (10^7/ml) in 5 ml of medium. In both instances the growth was measured for 72 hours.

Growth on solid media. Bacteria were grown for 48 hours on agar, washed off with saline and after three subsequent washings resuspended into saline. The suspension was homogenized, diluted 10^{-1} — 10^{-7} and 0.1 ml of respective dilutions was spread on the surface of appropriate media in duplicates. Controls were grown on blood agar with 10 % sheep blood. Both aerobic and anaerobic cultivations (Fortner's method) lasted 48 hours.

Evaluation of growth. Growth was measured in fluid media turbidimetrically on LP photometer with red filter. Results were expressed as mean values obtained from 10 samples, cultivated simultaneously. The growth in Erlenmeyer flasks with an attached test tube was measured in the same way. All samples were checked for purity.

On solid media colony forming units were counted and titres obtained on different media with different haemin concentrations were compared with the control on blood agar medium.

Preparation of the particulate fraction for the cytochrome assay. The method of Pappenheimer et al. (1962) and of Kusaka et al. (1964) was used. Twice washed suspension of bacterial bodies was mechanically desintegrated (Novotný 1964) with glass beads for 8 minutes, filtered through sintered filter Nr 1 to separate glass beads and gross fragments of bacteria. The filtrate was centrifuged at 10^4 g for 40 minutes (MSE SUPER-SPEED centrifuge), and the supernate was recentrifuged at 105,000 g for 30 minutes (Janetzki VAC 60). Pink sediment was washed with distilled water, recentrifuged and resuspended in 6 ml of phosphate buffer pH 7.2. The opalescent fluid was used for spectrophotometric analysis. Turbid samples had to be diluted.

Evaluation of cytochromes. To a cuvette with 3 ml of particulate fraction sodium dithionit was added and mixed thoroughly. The spectrophotometric assay was performed on an automatic device (Optica Milano, or Beckman). Sensitivity

was adjusted according to the turbidity of the sample. The assay was run 15 minutes after the addition of sodium dithionit, as at a longer time interval proteins start to precipitate. Extinctions of oxyd/red differential spectrum in the range 400 nm—610 nm were recorded.

RESULTS

Todd-Hewitt broth with serum, enabling good growth of bacteria, was chosen for growth experiments. All attempts to use defined synthetic medium (e.g. casein hydrolysate with vitamines Wilson 1954) gave irreproducible results. The result of our first experiment is shown in figure 1. Though bacteria grow even in the absence

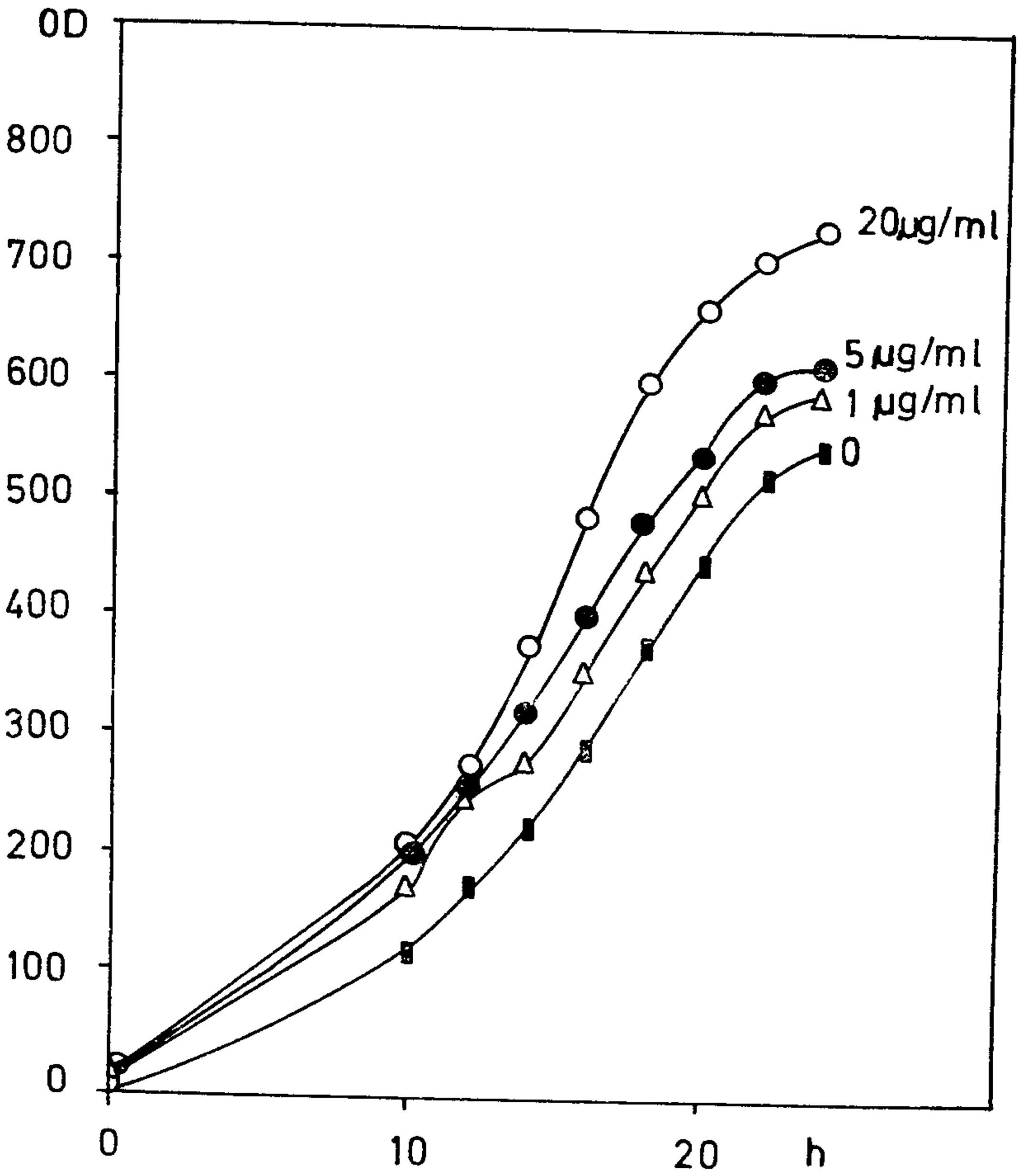

Fig. 1 — Growth curves of Corynebacterium haemolyticum at different haemin concentration. Todd Hewitt broth with 1, 5 and 20 mcg/ml of haemin. Optical density (OD) as mean value from ten parallel test tubes.

of haemin, its influence is clearly demonstrated. The background growth can be explained by natural porphyrines occurring in the medium as remnants of blood in beef hearts, or in calf serum. For this reason further experiments were done on solid media. Colonies grown on minimal media at aerobic conditions were smaller than those on control media. Colonies appearing on media without haemin either aerobic or anaerobic condition were very minute. Media containing haemin (10 mcg/ml or more) showed normal growth with colonies of the same size as of those grown onblood agar.

Growth at anaerobic conditions is substantially lower in absence of haemin. The growth enhancement depends on the concentration of haemin. At anaerobic conditions bacteria grow better at 5 mcg/ml of haemin reach the optimum (Fig. 2).

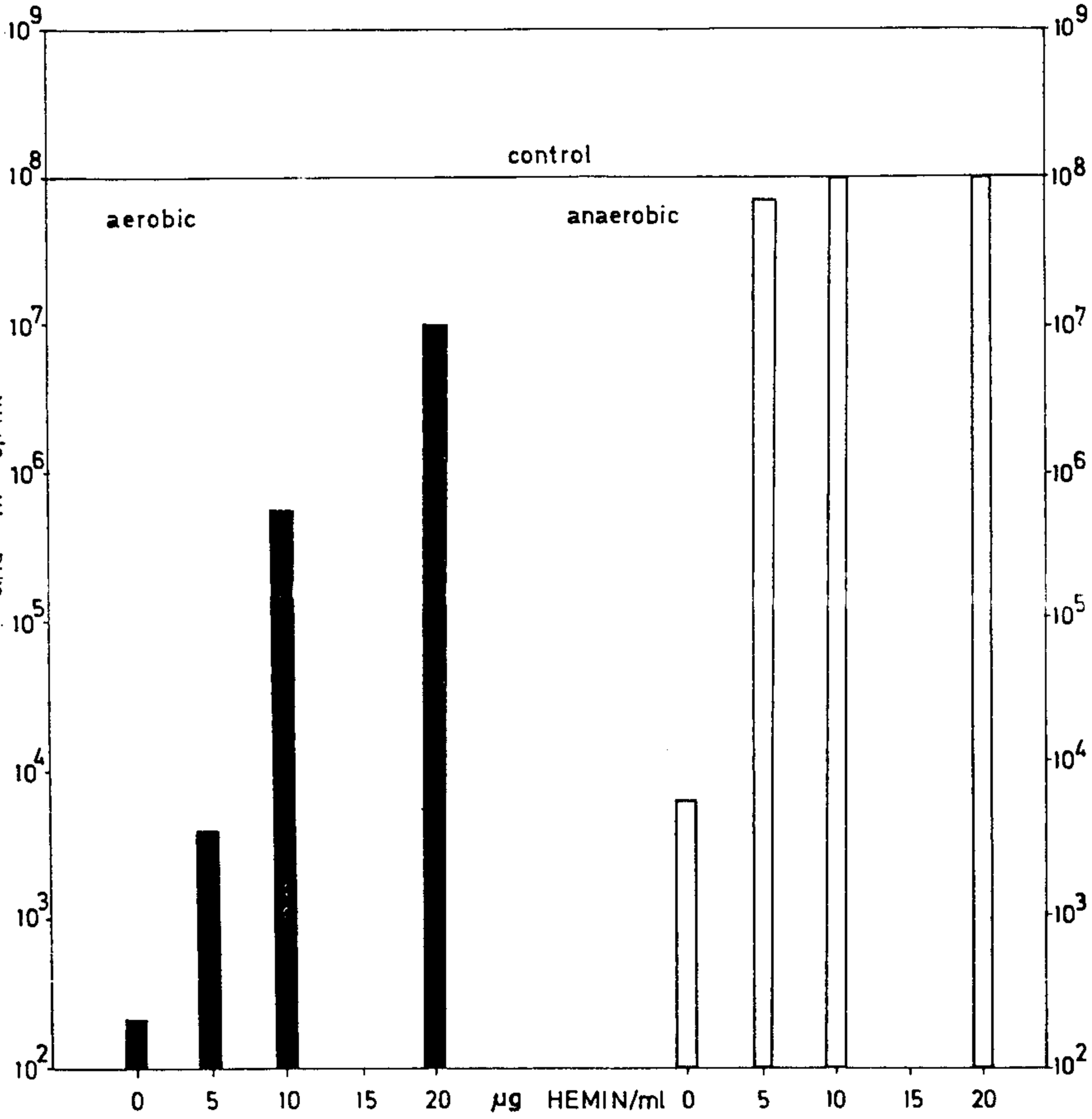

Fig. 2 — Growth of colonies on TSB solid medium at aerobic and anaerobic conditions. Control grown on blood agar.

As it is known, that haemin is structural analogue of the prosthetic group of cytochrome b_1, we tried to find out whether the addition of haemin leads to an increase in cytochrome content. Cytochrome b_1 increased even after the addition of hemoglobin. This increase occurs against control on TH broth. In this instance blood substance present in basal medium is considered as the source of porphyrine (Fig. 3.).

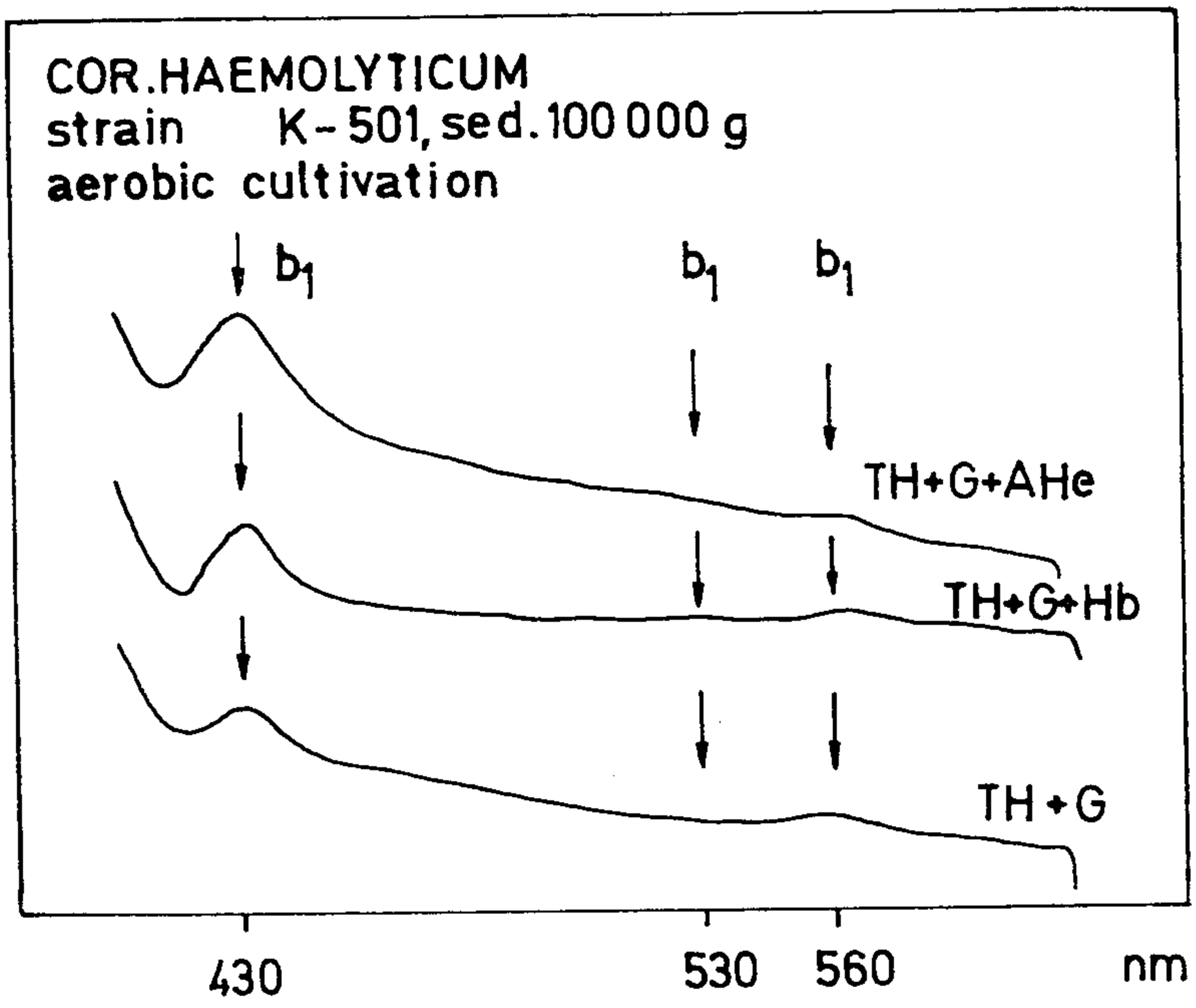

Fig. 3 — Differential oxid/red spectrum of the particulate fraction of Corynebacterium haemolyticum endoplasmatic material. Corynebacterium haemolyticum grown in Todd Hewitt broth and glucose (TH + G) with haemoglobin (10 mcg/ml) (TH + G + Hb) or haemin 10 mcg/ml added (Th + + G + AHe)

For the expression of cytochrome b_1 content as linear function of haemin concentration at aerobic conditions the same media as in preceding experiments were used. Particulate fraction of control cells containing cytochrome b_1 was diluted to show a minimal peak at 430 nm at maximal sensitivity. Particulate fractions obtained

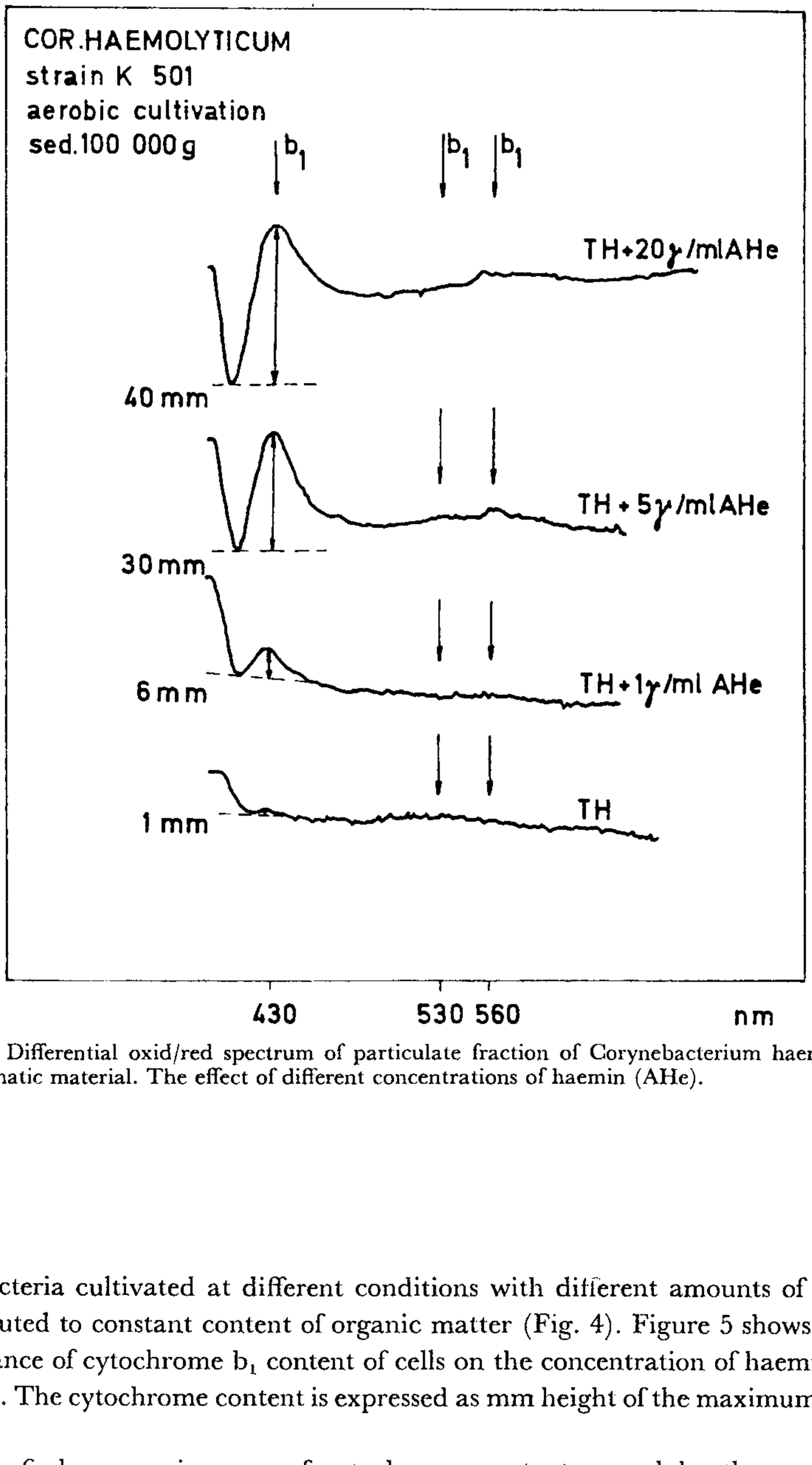

Fig. 4 — Differential oxid/red spectrum of particulate fraction of Corynebacterium haemolyticum endoplasmatic material. The effect of different concentrations of haemin (AHe).

from bacteria cultivated at different conditions with different amounts of haemin, were diluted to constant content of organic matter (Fig. 4). Figure 5 shows a linear dependance of cytochrome b_1 content of cells on the concentration of haemin in the medium. The cytochrome content is expressed as mm height of the maximum 430 nm peak.

Figure 6 shows an increase of cytochrome content caused by the presence of haemin 10 mcg/ml in the medium at anaerobic cultivation.

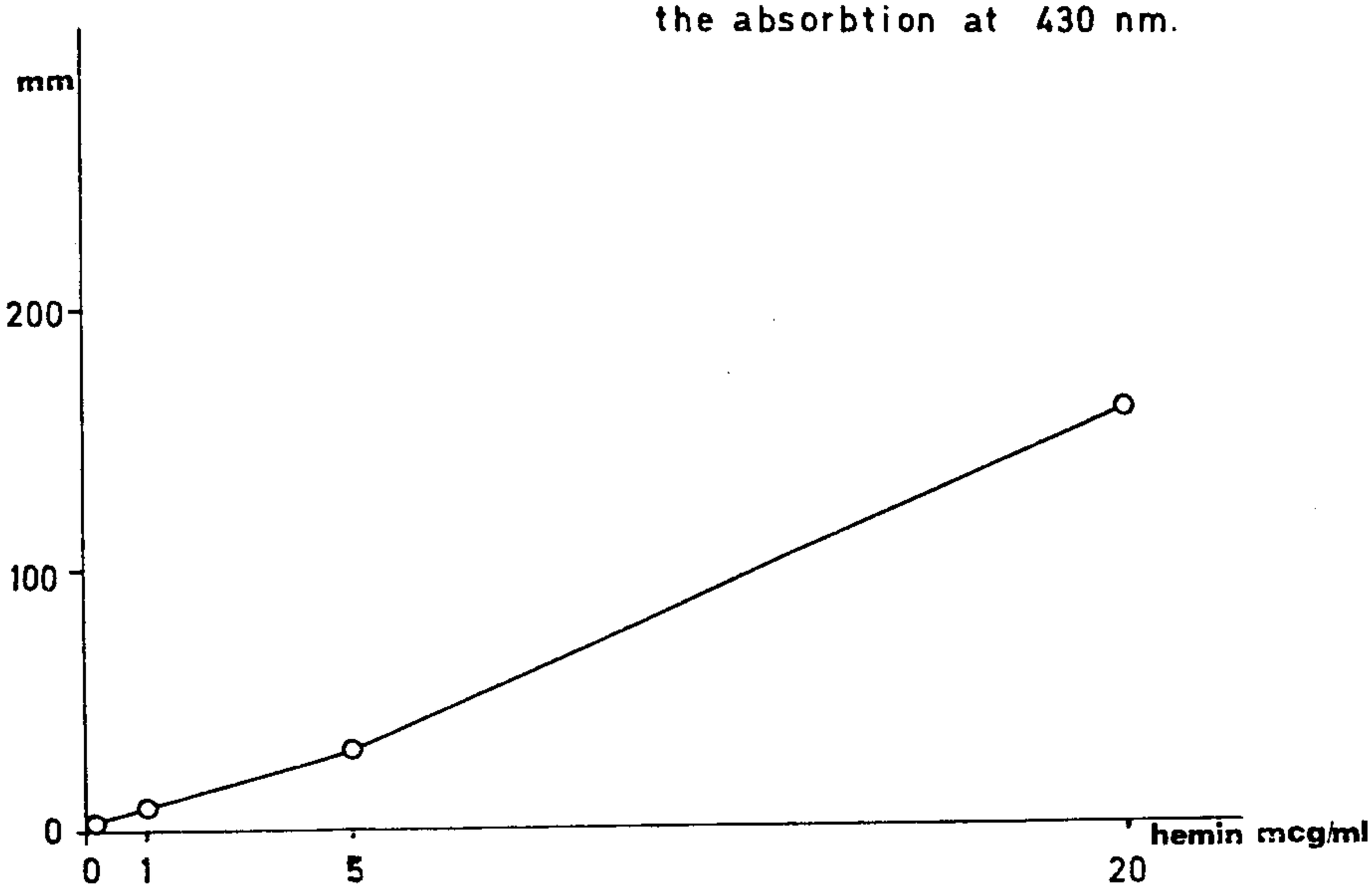

Fig. 5 — 430 nm peak height plotted against haemin concentration. Values taken from fig. 4.

DISCUSSION

From the results presented here a conclusion may be drawn, that haemin is a growth factor for vegetative cells of Corynebacterium haemolyticum, especially at aerobic conditions. The increase in bacterial density is proportional to the concentration of haemin in the range of 1 mcg/ml—20 mcg/ml. In some experiments distinct growth was observed only if haemin was added, whereas the growth in the basal medium (TH broth) was minimal. This maight be explained by the content of natural porphyrines in media prepared from raw meat and a beef heart, which cannot be controled properly. At anaerobic conditions attainable by the classical Fortner method a good growth can be obtained in the absence of both haemin and natural porphyrins. It reaches its optimum after addition of at 5 mcg/ml of haemin.

Biberstein and Gills (1961) reported that the growth of certain strains of Haemophilus species depends on the concentration of haemin in the range of 1 mcg/ml—

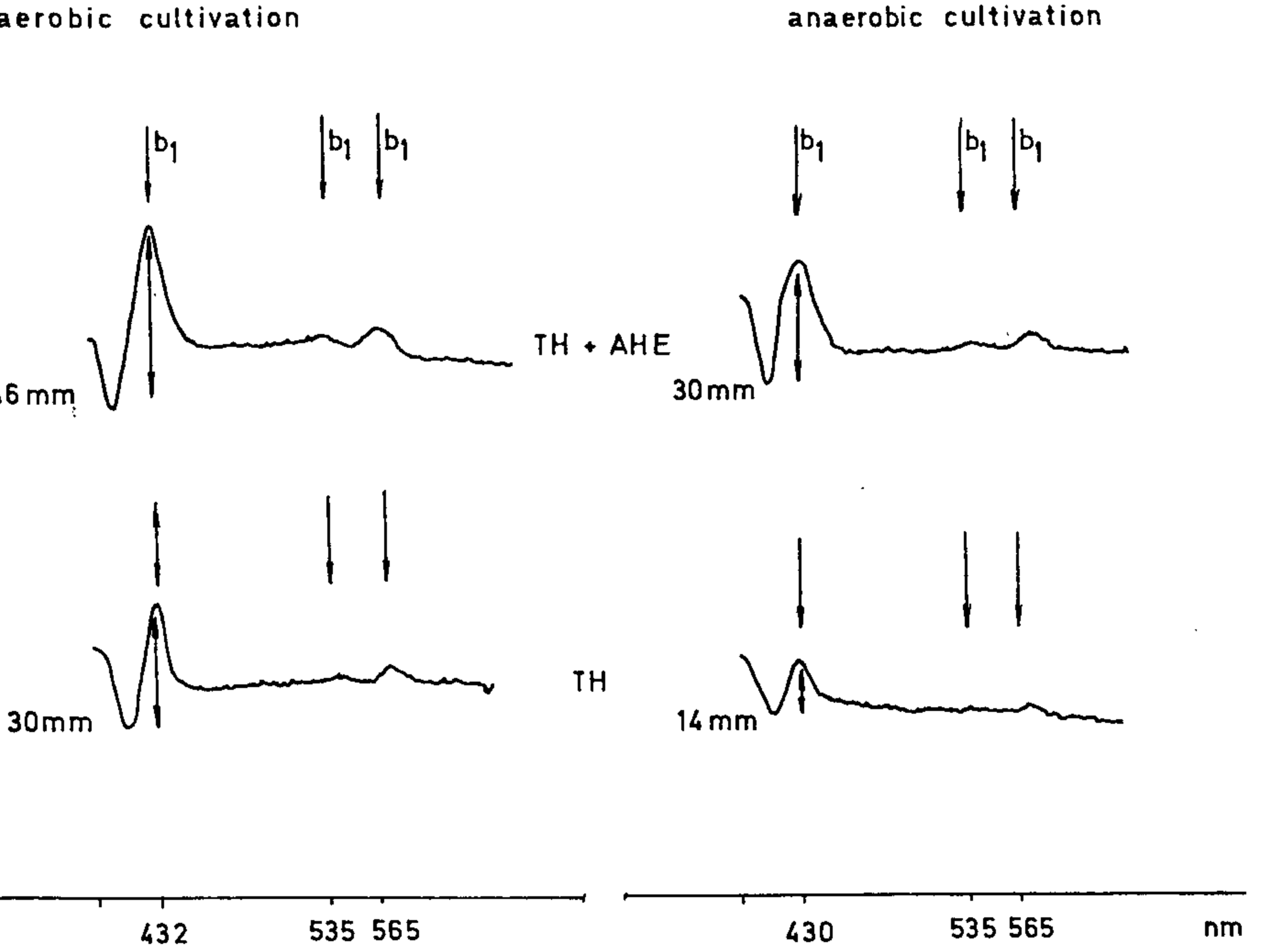

Fig. 6 — Oxid/red differential spectrum curves of bacteria with haemin at both aerobic and anaerobic conditions. Todd-Hewitt broth (TH), Todd-Hewitt broth with hemin (TH + AHe).

10 mcg/ml at aerobic conditions. Chang and Lascelles described mutants of Staphylococcus aureus, dependent on haemin at aerobic conditions. Though the microbes were able to synthetize the protein moiety of the cytochrome b_1 molecule in the absence of haemin they were unable to grow.

In about 50 % of haemophilus strains under study, the growth dependency of haemin was associated with catalase formation. On the other hand staphylococcal mutants were able to reduce nitrates and to synthetize cytochrome b_1 at anaerobic conditions. The authors suggest the following electron transport for these mutants:

Lactate → NAD → Flavine → Cytochrome b_1 → Nitrate-reductase → NO_3 → NO_2

Earlier we have demonstrated, that washed suspension of Corynebacterium haemolyticum neither produces catalase nor reduces nitrates (Patočka et al. 1962). Thus

it resembles the animal Corynebacterium pyogenes and differs from typical corynebacteria (Corynebacterium diphtheriae, Corynebacterium ovis, Corynebacterium ulcerans).

However, Robinson (1966) found catalase activity in the endoplasmatic material after electrophoretic separation.

Cytochrome b_1 was found in the particulate fraction as the only cytochrome present. Thus we can draw a parallel with the mentioned haemophilus strains and with some mutants of Staphylococcus aureus isolated by Jensen (1967). These mutants were protoporphyrine dependent at aerobic conditions. They showed respiration catalase activity, and grew only in the presence of porphyrines. The similarity with mutants of Staphylococcus aureus showing nitrate reductase activity cannot be proved. Nevertheless it can be presumed, that cytochrome b_1 formed even at anaerobic conditions transfers electrones on another anorganic acceptor. We have shown, that Corynebacterium haemolyticum needs haemin both at aerobic and anaerobic conditions. The formation of minute atypical colonies at aerobic and anaerobic conditions on media without porphyrines suggest two classes of mutants — one haemin dependent the other independent.

SUMMARY

1. Growth of colonies of Corynebacterium haemolyticum at aerobic conditions is haemin- dependent.

2. This dependence is expressed to a lesser extent at anaerobic conditions.

3. Both haemin and haemoglobin enhance at anaerobic or aerobic conditions production of cytochrome b_1 detected in the particulate fraction of the cytoplasmatic material.

4. The way cytochrome b_1 might function in electron transport in bacteria and its relation to catalase and to nitrate reductase is discussed.

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