The Antibacterial Activity of Haematin

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SUMMARY: Haematin in concentrations of 1/1,000,000-1/2,000,000 inhibited the growth of a number of bacteria most of which were aerobic sporing bacilli. Deutero-haematin and mesohaematin were also inhibitory, but not haematohaematin, urohaematin and a number of iron-free porphyrin derivatives. In a few cases whole blood was inhibitory, but generally only trypsin-digested laked red cells. The mechanism of the antibacterial activity is not understood.

This work arises from an observation by Dr R. L. Vollum that a culture of *Bacillus subtilis* appeared to autolyse very readily when grown on a blood-agar plate. It was thought that this observation might throw light on the mechanism of bacterial autolysis, but further investigation showed that autolysis was only a secondary result of the antibacterial action of haematin liberated from the blood.

These are not the first observations on the antibacterial activity of iron-porphyrins. While this work was in progress Professor Rimington pointed out that many years ago Kämmerer (1914) made the interesting observation that mesohaematin (i.e. haematin with the two vinyl groups reduced) was inhibitory to a large number of Gram-positive bacteria, including aerobic sporing bacilli, staphylococci, streptococci, pneumococci and corynebacteria. *B. anthracis* was inhibited by concentrations of 1/500,000. He believed that the antibacterial action of mesohaematin exceeded that of many antiseptics such as Karbol and Lysol, and was comparable with that of corrosive sublimate. Kämmerer did not observe any antibacterial activity of haematin, except in concentrations as high as 1/800; at 1/2,000 it tended to stimulate the growth of his test organisms.

Glass (1939) observed that haematin at a concentration of 1/100,000 was inhibitory to certain *mitis* strains of *Corynebacterium diphtheriae*.

METHODS

Bacterial growth in liquid cultures was determined by measuring the optical density (D) of the cultures with a photoelectric densitometer. D is the logarithm of the ratio of the incident light to the light transmitted through 1 cm. of the bacterial suspension. The values for D were translated into mg. dry weight organism/ml. by means of an experimentally determined standard curve. Corrections were made for the optical densities of uninoculated media. The cultures were grown in shallow layers of medium which were continuously agitated.

Proteolytic activity of bacterial cultures was determined in gelatin stabs and also by stabbing cultures into a 2% suspension of casein in nutrient agar

poured in Petri dishes. After 48 hr. incubation proteolytic colonies were surrounded by clear transparent zones in a white opaque matrix of undigested casein.

Succinic oxidase activity of washed cultures was judged by measuring the reduction time of methylene blue in the presence of succinate in Thunberg tubes. Bacterial respiration was measured in the usual way with Warburg manometers. pH values were determined electrometrically with a glass electrode.

RESULTS

Effect of blood on the growth of aerobic sporing bacilli

Petri dishes were poured with nutrient agar and after cooling a half-circle of the agar was removed and replaced by agar containing 1.5 % horse blood. The plates were then spread with an inoculum of *B. subtilis* and incubated at 37°. After 12 hr. incubation the organism had grown lightly and evenly on both halves of the plate. After 24 hr. the organisms on the blood agar were completely autolysed while the growth on the other half of the plate was profuse and normal. The organisms on this half eventually autolysed after 4–6 days. Whole blood could be replaced with washed red cells, which were more effective when laked with distilled water. Plasma had no effect. The effect with whole red cells could be obtained with only 6 out of 35 strains of *B. subtilis* that were tested, and with none of 19 strains of *B. licheniformis*. With laked red cells a few more strains of *B. subtilis* were affected.

When trypsin (25 µg. crystals/ml.) was incorporated in the laked red cell agar the effect was changed qualitatively and quantitatively. Instead of growing and then rapidly autolysing, the organism failed to grow at all. Moreover, inhibition of growth was now caused by a much lower concentration of laked red cells and affected all 35 strains of *B. subtilis* and all 19 strains of *B. licheniformis*. Control experiments showed that trypsin itself had no observable effect on these organisms. Strains of *B. subtilis* fell into at least four groups (Table 1) as far as their behaviour towards red blood cells was

Table 1. *Differences in inhibition of growth of different groups of strains of B. subtilis by treated and untreated red cells*

Group of <i>B. subtilis</i> strains	Growth inhibited by			
	whole red cells	red cells + trypsin	laked cells	laked cells + trypsin
I	+	+	+	+
II	—	+	—	+
III	—	—	+	+
IV	—	—	—	+

+ = inhibition

concerned, but they were not always consistent in their behaviour; after being stored in the cold for several months strains from group IV behaved like those from group II. The differences in behaviour of groups I and IV

could be due to differences in their sensitivity to the inhibitory substance, or to differences in their production of proteolytic enzymes. Strains from groups I and IV were tested for their sensitivity to the inhibitory substance (which had been shown to be haematin) and they did not differ markedly, nor was there any obvious difference in their proteolytic activity against gelatin or casein. On the other hand, strains of *B. licheniformis*, which all behaved towards red cells like group IV *B. subtilis* strains, were less proteolytic towards these substrates than *B. subtilis*. It is difficult to see how differences in sensitivity or protease production could account for the fact that laked cells were more effective than trypsin-treated cells for strains of groups I and III, and less effective for group II. These differences are also apparent in Pl. 1, where in dish 1 the inhibition zone at D (laked cells) is greater than that at C (whole red cells + trypsin), but in dish 2 the reverse is true.

Some group IV strains were haemolytic, but they were inhibited only by laked red cells + trypsin, and not by haemolysed cells + trypsin. A possible explanation for this is that the cells were not haemolysed until the organisms were fully grown and therefore less sensitive to the action of haematin.

Pl. 1, dish 2, suggests that red blood cells contain a slowly diffusible stimulatory substance as well as a more rapidly diffusible inhibitory substance. There is growth on all the plugs, but those in which the inhibitory substance has been released by trypsin are surrounded by zones of inhibition. In the plugs themselves the concentration of stimulatory substance is sufficient to counteract the inhibitory substance.

It seems clear that the effect of blood on *B. subtilis* is not directly to cause it to autolyse, but to inhibit its growth. When intact red cells are used the inhibitory substance is released slowly, so that the organism has time to grow. But after a time a sufficient concentration of the inhibitory substance is released to stop the growth of the organisms, which then autolyse. It is known that *B. subtilis* autolyses readily when its growth ceases. When the inhibitory substance is released from laked cells by trypsin the organisms are inhibited immediately and they fail to grow.

The antibacterial activity of haematin

Since the inhibitory substance was liberated from red cells by proteolytic enzymes, and since concentrates of the substance were dark-coloured it was thought that it might be haem. Haemin was, therefore, prepared from laked ox blood by the method of Fischer & Orth (1937) and purified. The purified product was dissolved in dilute NaOH and its inhibitory effect tested on cultures of *B. subtilis* in potato dextrose medium (Arriagada *et al.* 1949) at pH 7.5 (where it is in the form of haematin). A concentration of 1/2,000,000 inhibited the growth of an inoculum (loopful of an overnight culture) of *B. subtilis*, but at this concentration the inhibition was only temporary and amounted to a prolongation of the lag-phase by several hours. At 1/1,000,000 it generally, but not always, prevented an inoculum from growing. Higher concentrations of haematin were needed to inhibit growing cultures (Fig. 1).

To observe the full effect of haematin it was necessary to avoid heating it in the presence of complex media. When it was autoclaved in heart broth or heart agar the concentration that was inhibitory to an inoculum of *B. subtilis* generally rose to about 1/40,000, and sometimes to 1/10,000. Autoclaving haematin in the presence of synthetic media had a much smaller effect.

Fig. 1. Inhibition of growth of *B. subtilis* in potato glucose medium by haematin. Curve 1, 1/2,000,000 haematin added before inoculation; Curve 2, 1/1,000,000 haematin added before inoculation; Curve 3, 1/250,000 haematin added 3.5 hr. after inoculation; Curve 4, 1/250,000 haematin added 5.5 hr. after inoculation.

The effect of haematin on other aerobic sporing bacilli was tested by streaking them (3 or 4 at a time) on plates containing adjacent half-circles of plain heart agar and heart agar containing 1/30,000 haematin (added to the molten agar at 47°). The results of these experiments are shown in Table 2. In a preliminary report (van Heyningen, 1948) it was stated that *B. anthracis*, *B. laterosporus*, and *B. alvei* were not inhibited by 1/20,000 haematin, but in those experiments the haematin was autoclaved in the nutrient agar.

At a concentration of 1/20,000 haematin did not inhibit the following

organisms: *Bacterium lactis aerogenes*, *Bact. coli*, *Bact. friedländeri*, *Chromobacterium prodigiosum*, *Micrococcus tetragenus*, *Mycobacterium phlei*, *Mycobact. smegmatis*, *Proteus vulgaris*, *Salmonella paratyphi* A & B, *Salm. typhi* H & O, *Sarcina lutea*, *Shigella flexneri*, *Sh. sonnei*, *Sh. shigae*, *Staphylococcus albus*, *Staph. aureus*, *Vibrio cholerae*, El Tor vibrio. At a concentration of 1/20,000 haematin completely inhibited two *mitis* strains and one *gravis* strain of *C. diphtheriae*, and partially inhibited one strain each of *C. hofmanni* and *C. xerosis*. On the other hand, it strongly stimulated the growth of two strains of starch-fermenting, gelatin-liquifying corynebacteria.

Table 2. *Inhibition of aerobic sporing bacilli by haematin (1/30,000)*

Organism	Number of strains tested	Completely inhibited	Partially inhibited	Not inhibited	Growth on control agar
<i>B. alvei</i>	8	8	0	0	+
<i>B. brevis</i>	12	7	4	1	+
<i>B. circulans</i>	10	7	3	0	+
<i>B. laterosporus</i>	3	3	0	0	+
<i>B. licheniformis</i>	19	19	0	0	++++
<i>B. macerans</i>	3	3	0	0	+
<i>B. polymyxa</i>	1	1	0	0	+
<i>B. pumilus</i>	5	5	0	0	++++
<i>B. subtilis</i>	35	35	0	0	++++
<i>B. anthracis</i>	2	0	2	0	++++
<i>B. megatherium</i>	7	0	7	0	++++
<i>B. carotarum</i>	7	0	2	5	++++
<i>B. cereus</i>	16	1	2	13	++++
<i>B. firmus</i>	1	0	0	1	++++
<i>B. fusiformis</i>	7	0	3	4	++++
<i>B. mycoides</i>	5	0	0	5	++++

The effect of a number of porphyrin and iron-porphyrin derivatives at a concentration of 1/20,000 was tested on *B. anthracis*, *B. brevis*, *B. cereus*, *B. licheniformis*, *B. megatherium* and *B. subtilis*, with the results shown in Table 3. These results suggest that mesohaematin is the most active of the compounds tested and that the presence of an iron atom in the molecule is essential for inhibitory activity. They also lead to the tentative conclusion that a non-polar group in positions 2 and 4 is necessary for inhibitory activity.

Keilin & Hartree (1947) found that deuterohaematin, haematin and haematoxaematin at a concentration of 2×10^{-4} M almost completely inhibited the oxidation of succinic acid by highly active muscle preparations. The enzyme could be protected from this inhibitory effect by the prior addition of glyoxalin or denatured protein and they concluded that the inhibition was due to the formation of a co-ordination compound between the haematin iron and some groups in succinic dehydrogenase important for its activity. In view of this the effect of haematin (1/5,000) on the succinic oxidase systems of washed suspensions of *B. subtilis*, *B. cereus*, *B. licheniformis* and *B. mycoides* was tested, but no conclusive results were obtained. Inhibition of oxidase

Table 3. *Effect of porphyrins and iron-porphyrins at a concentration of 1/20,000 on the growth of certain aerobic sporing bacilli*+ = complete inhibition; $\pm$ = partial inhibition; -- = no inhibition.

Porphyrin derivative*	Groups in positions 2 & 4	Inhibitory to:					
		<i>B. anthracis</i>	<i>B. brevis</i>	<i>B. cereus</i>	<i>B. licheniformis</i>	<i>B. megatherium</i>	<i>B. subtilis</i>
deuterohaematin	-H	±	+	-	+	±	+
haematin	-CH=CH ₂	±	+	-	+	±	+
mesohaematin	-CH ₂ -CH ₃	+	+	±	+	±	+
haematohaematin	-CHOH-CH ₃	-	-	-	-	-	-
urohaematin	-CH ₂ -CH(COOH) ₂	-	-	-	-	-	-
protoporphyrin	-CH=CH ₂	-	-	-	-	-	-
mesoporphyrin	-CH ₂ -CH ₃	-	-	-	-	-	-
haematoporphyrin	-CHOH-CH ₃	-	-	-	-	-	-
coproporphyrin	-CH ₂ -CH ₂ -COOH	-	-	-	-	-	-

* All these derivatives have -CH₃ groups in positions 1, 3, 5 and 8, and -CH₂-CH₂-COOH groups in positions 6 and 7, excepting urohaematin which has -CH₂-CH(COOH)₂ groups.

activity was observed, but it was very variable, and the organisms (*B. subtilis* and *B. licheniformis*) whose growth was inhibited by haematin were no more affected than those (*B. cereus* and *B. mycoides*) whose growth was not inhibited.

The effect of haematin on the respiration of these organisms was also examined. No inhibition of respiration was observed with haematin at a concentration of 1/50,000, but at 1/5,000 inhibition was complete, whether

FIG. 2. Effect of haematin (1/5,000 final concentration) on respiration of organisms whose growth is inhibited by haematin (*a* & *b*) and whose growth is not inhibited by haematin (*c* & *d*). Haematin added at times indicated by arrows.

or not the growth of the organisms was inhibited by haematin. The only difference that could be seen was that with organisms whose growth was inhibited (*B. licheniformis* and *B. subtilis*) there was a lag period of about 15 min. between the addition of the haematin and the cessation of respiration, whereas with those whose growth was not inhibited (*B. cereus* and *B. mycoides*) the inhibition was immediate (Fig. 2). Superficially, at any rate, this was the reverse of what might have been expected. In control experiments the respiration of *Bact. coli* was unaffected by 1/5,000 haematin.

Dish 1

Dish 2

DISCUSSION

The results reported in this paper confirm Kämmerer's interesting observations in so far as mesohaematin was found to be antibacterial and to have a greater activity than haematin. It is possible that the inactivity of haematin in Kämmerer's experience may have been due to his autoclaving this substance in his nutrient medium.

Anigstein, Whitney & Micks (1950) (see also Whitney, Anigstein & Micks, 1950) reported that tryptic digests of ticks are inhibitory to a number of species of bacteria, the aerobic sporing bacilli being the most sensitive. The antibacterial activity of extracts of ticks engorged with blood was considerably enhanced, and the inhibitory substance was shown to be associated with red blood cells. They suggest that the inhibitory substance is a peptide, but it is more likely to be haematin.

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EXPLANATION OF PLATE

Pl. 1. Differences in inhibition of two strains of *B. subtilis* by red cells. Holes in nutrient agar plugged with agar containing: A, 25 μ g. trypsin/ml.; B, 3% washed horse red cells; C, 3% red cells + 25 μ g. trypsin/ml.; D, 3% laked red cells; E, 3% laked red cells $\times$ 25 μ g. trypsin/ml.

Dish 1 flooded with inoculum from group I (see Table 1).

Dish 2 flooded with inoculum from group II.

The agar in both dishes contained 0.03% Neutral Red in order to simplify the photography of dishes containing red plugs. In dish 1 some of the Neutral Red has crystallized out.

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