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ON THE USE OF FLOW MICROCALORIMETRY IN ESTIMATING THE BIOLOGICAL EFFECT OF ADVERSE SUBSTANCES ON E. COLI
IV. AN INVESTIGATION ON 5 TEST SUBSTANCES*

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ABSTRACT

The effect of five test substances of different chemical nature on the growth of E. coli was investigated by flow microcalorimetry. The concentration of the test substances in the broth was 15 mmol/l, uniformly. The test substances were added during the logarithmic growth phase at a heat flow of 40 µW/ml.

Compared to the control culture acetone and trimethylamine decrease the maximum heat flow only to an insignificant degree. Formaldehyde decreases the production of the culture, distinctly. When adding m-cresol and p-cresol high peaks indicating strong heat effects are observed. As far as the test substances acetone, trimethylamine and formaldehyde are concerned flow microcalorimetric findings agree with the results obtained from bacterial count and optical density. As far as m- and p-cresol are concerned high heat flows stand opposite to decreasing germ numbers in the broth. It is supposed that adsorption and desorption phenomena in the calorimetric tubes contribute to the extended heat production when testing these substances.

INTRODUCTION

Modern flow microcalorimeters (ref.1) are used successfully to test the action of antibiotics on growth and metabolism of bacteria (e.g. ref.2, ref.3, ref.4) and yeasts (e.g. ref.5, ref.6). The antibiotics are usually added during the logarithmic growth phase of the culture (ref.3, ref.4). Microcalorimeters have a pretention for automation and can be used in diagnostic medical microbiology (ref.7). The method is fast, sensitive and reliable. It seems that the opacity of test solutions as well as suspended materials or emulsions do not present problems (ref.8, ref.9). Reports on the investigation of other chemical compounds are scarce. A few investigations exist on disinfectants (ref. 9), heavy metals (ref. 10) and organic compounds (ref.11) using as test cultures respiring E.coli cells in glucose phosphate buffer (ref. 9) under aerobic and anaerobic conditions (ref.10) or resting cells of

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E. coli in grown-through nutrient broth (ref.11).

This article reports on experiences when adding test compounds of different chemical nature into the logarithmic growth phase of a E. coli culture. The aim is to find out whether flow microcalorimetry is suitable to characterize the biological effect of chemicals in a similar effective way as applies to antibiotics. The test compounds are used in aquaeous solution at a concentration of 15 mmol/l. Parallel investigations on the germ content and on the optical density of the broth are carried out.

MATERIALS AND METHODS

A flow microcalorimeter of the heat conduction type (ref.12) equipped with a flow-through-chamber of a volume of 0.7 ml was used. The sensitivity of this detector was found to be 59.1 μV/mW. The culture was prepared and grown outside the microcalorimeter in a growth vessel at 37°C to which the test substances were added. From this vessel the broth was pumped through the calorimeter at a constant flow rate of 15 ml/h and reached the detector in the microcalorimeter about 8 min later. The resulting power-time-curves were recorded by means of a compensation recorder type R-16 (Rikadenki Electronics, Freiburg, FRG).

The E. coli strain ATCC 11229 was used as test organism. The standard nutrient broth no.CM 67 was purchased from Oxoid, Wesel. Test substances of the purest possible quality were bought from Merck, Darmstadt. All solutions were prepared in sterilized, distilled water.

Initially, the broth was inoculated with E. coli resulting in a concentration of bacteria of about 2×10^5 CFU/ml (CFU = colony forming unit). The test substances were added during the logarithmic growth phase of the culture, when the power-time-curve indicated a heat production of 40 μW/ml. In order to keep up a constant volume of broth in the growth vessel until adding the test substance a sufficient volume of broth was pumped continuously into the growth vessel from a supply vessel by means of a peristaltic pump. After the addition of the test substance this pump is switched off. The growth vessel contains 200 ml of broth and the heat production can be monitored up to 6 h, at least. Further details of the design of the growth vessel were given in a previous paper (ref.13).

The bacterial count was performed as viable count by the agar plate count technique. The bacterial solutions were diluted and plated with spatula to petri dishes containing nutrient agar no. CM 463 (Oxoid, Wesel, FRG). After incubation for 24 h at 37°C the colonies were counted with the aid of an electronic colony counter model 600 (Fisher Instruments, Farmingdale, N.Y.,USA).

The optical density was monitored photometrically by pumping the bacterial

broth continuously (15 ml/h) from the growth vessel through a micro-flow-through-cuvette (type 4052, Eppendorf, Hamburg, FRG). The broth reaches the photometer almost at the same time as the calorimeter.

All tests were run twice. Mean values are given representing a deviation of not more than ±10%, in most of the cases less than ±5%.

RESULTS

Figure 1 shows examples of the power-time-curves of E. coli in nutrient broth without (control) and with the addition of 15 mmol/l of formaldehyde and p-cresol, respectively. The addition took place during the logarithmic growth phase at a heat production of 40 µW/ml. Compared to the control the addition of formaldehyde diminishes the height of the power-time-curve, drastically. Following the addition of p-cresol the power-time-curve increases rapidly up to more than 200 µW/ml and then sharply decreases to only a few µW/ml. The highest point of the power-time-curve is reached distinctly earlier than in the control curve.

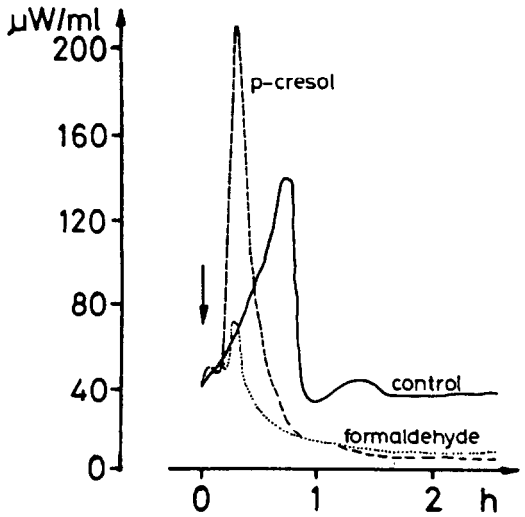

Fig. 1: Power-time-curves of E. coli in nutrient broth without (control) and with the addition of 15 mmol/l of formaldehyde (....) and p-cresol (---). The arrow (↓) indicates the injection of the test substance at a heat flow of 40 µW/ml.

Figure 2 gives the heat flow in µW/ml at the instant when the test substances are added (1) and 10 min (2), 30 min (3), 60 min (4) and 120 min (5) later. Additionally, the highest peak of the power-time-curve is marked indicating the maximum heat flow (H). In the control the maximum heat flow is

reached about 45 min after time 1. The peak is followed by a sharp decrease to about 20 µW/ml. Under the influence of the test substances the maximum heat flow is reached already within 30 min after time 1.

When comparing the amounts of the maximum heat flows large differences are observed. Acetone and trimethylamine show maximum heat flows comparable to the control. 120 min after adding trimethylamine a second increase in heat production is observed indicating a repeated biological activity. In some extent this observation coincides with the bacterial count (see Figure 3). Formaldehyde diminishes the maximum heat production of the test culture about by half. After the addition of m-cresol and p-cresol high maximum heat flows are monitored followed by a sharp decrease to a few µW/ml within 60 min (4) and 120 min (5).

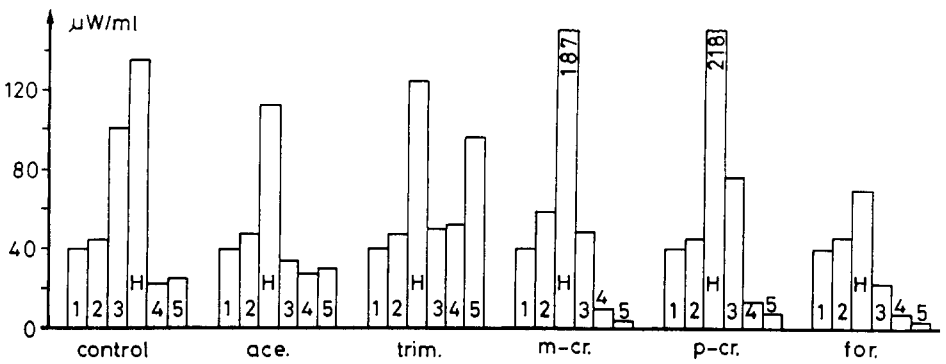

Fig. 2: Heat flow in µW/ml just before adding the test substance (1) and 10 min (2), 30 min (3), 60 min (4) and 120 min (5) later. ace = acetone, trim = trimethylamine, m-cr = m-cresol, p-cr = p-cresol, for = formaldehyde.

Figure 3 gives the germ content (CFU/ml) in the broth, estimated just before (1) and 10 min (2), 30 min (3), 60 min (4) and 120 min (5) after the addition of the test substances. The addition of acetone reveals no differences compared to the control. Under the influence of trimethylamine E. coli seems to grow slower than in the control. Trimethylamine shifts the pH-value of the broth to about 8.5. Probably, the akalinity influences the growth of the germs. A repeated increase of the bacterial count after 120 min is indicated. The germ content decreases distinctly under the influence of m-cresol, p-cresol and formaldehyde. 120 min after adding formaldehyde no living E. coli were

found in the broth. 60 min after adding p-cresol the suspension was free of colony forming units.

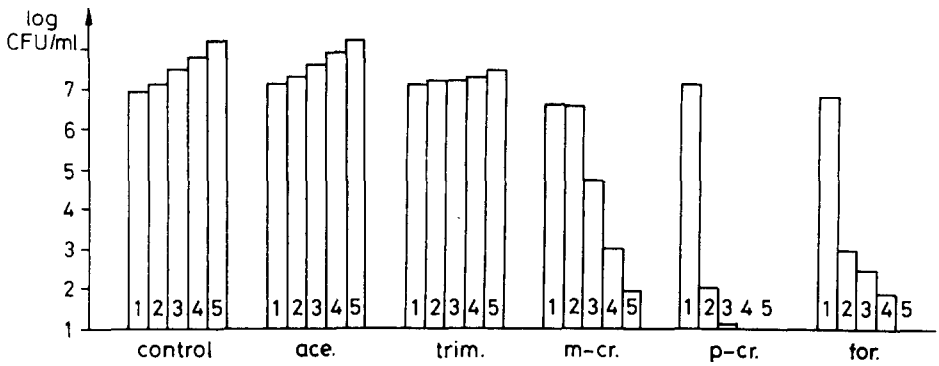

Fig. 3: Bacterial count in the medium just before (1) and 10 min (2), 30 min (3), 60 min (4) and 120 min (5) after the addition of the test substances. ace = acetone, trim = trimethylamine, m-cr = m-cresol, p-cr = p-cresol, for = formaldehyde, CFU = colony forming unit.

Figure 4 gives the example of the curves obtained by optical density measurement for control (A), trimethylamine (B), formaldehyde (C) and p-cresol (D). Under the influence of trimethylamine a small delay is observed as compared to

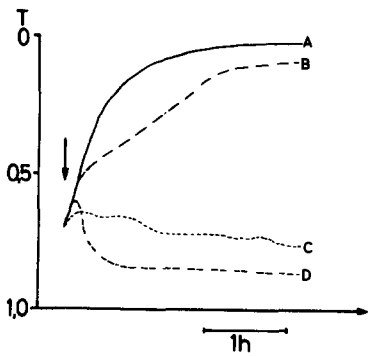

Fig. 4: Optical density of the broth inoculated with E. coli (A = control) after the addition of 15 mmol/l trimethylamine (B), formaldehyde (C) and p-cresol (D). The arrow (↓) indicates the injection of the test substance at a heat flow of 40 µW/ml. T = transmission.

the control. The density stagnates after the addition of formaldehyde and decreases after adding p-cresol. The curves obtained by adding acetone and m-cresol which are not depicted in Figure 4 are similar to the control and the p-cresol curve, respectively.

DISCUSSION

The results show that the highest heat flow is reached earlier under the influence of the test substances than in the control. The test compounds seem to accelerate the heat production of the test culture. Membrane-active compounds like phenol at certain concentrations can help to increase the transport of nutrients (and other compounds) into the cell causing an initial increase of the metabolic activity of the cell (ref.9). Membrane activity is known from the test substances m-, p-cresol and formaldehyde (ref.14). Possibly, similar mechanisms are effective as far as acetone and trimethylamine are concerned.

When comparing the amounts of heat produced (maximum heat flow) large differences between the test compounds are observed. The test substances acetone and trimethylamine do not significantly influence the maximum heat flow. A small influence of trimethylamine on the bacterial count and the optical density is observed, while acetone does not provide any reactions at the concentration used in this study. Acetone possesses some antibacterial properties on vegetative germs (ref.15). Our own experiences show that 5% to 10% acetone are necessary to diminish the germ content of E. coli in nutrient broth at 37°C during 24 h. The small influence of trimethylamine is probably due to its alkaline properties.

The antibacterial effect of formaldehyde is demonstrated by decreasing heat production, germ content and optical density. The minimum inhibitory concentration of formaldehyde is decribed at about 20 mg/l $\hat{=}$ 0.7 mmol/l (ref.14).

Problems arise with the interpretation of the heat production after adding m- and p-cresol. These test substances show pradoxical reactions. The heat production increases while the germ content and the optical density decreases or stagnates. Cresols are known as constituents of disinfectants (ref.16). They have antibacterial properties which are shown by the decreasing germ numbers and diminished optical density. The observed heat production must be caused by other processes. It is supposed that adsorption and desorption phenomena in the microcalorimetric tubes contribute to the extended heat production when testing these substances. This assumption is supported by Figure 5 demonstrating the sharp increase in heat production when adding 5.6 mmol/l m-cresol to pure, uncontaminated broth (1), and the sharp decrease in heat production when switching back to uncontaminated, pure broth (2).

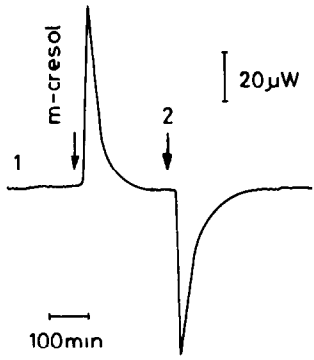

Fig. 5: Heat effects produced in a flow microcalorimeter by adding 5.6 mmol/l m-cresol to sterile, pure nutrient broth (1) and after switching back to sterile, pure nutrient broth (2)

The same reactions are obtained when using sterile water as medium to which the test substances are added or when mixing medium and test substance eight hours before the insertion into the microcalorimeter, already. In this case mixing reactions might only occur at the interface pure medium - medium with test substance in the microcalorimetric tubes. Former experiences with m-cresol, when testing its influence on respiring E. coli cells in glucose phosphate buffer, revealed similar results.

Possibly, the solubility of the test substances in water take an influence as well. Acetone is soluble in all proportions trimethylamine is very soluble and formaldehyde is rather soluble in water, while m- and p-cresol are only slightly soluble (ref.17). Perhaps, the difficulties can be diminished when using a parallel twin-flow-microcalorimeter (ref.18) for compensating the unwelcome heat effect by a germ-free control to which only the test substance is added.

In spite of these problems, flow microcalorimetry is a superior technique for monitoring the metabolic heat production of bacterial cultures and the action of metabolic modifiers on the bacterial growth, continuously. A great advantage is the short assay time. Recent experience indicates that microcalorimeters of the heat conduction type in connection with a chemostat are suitable to observe and to quantify the biological action of some pollutants in surface water on pure bacterial cultures (ref.19). In the present study good examples are given when testing the compounds formaldehyde, acetone or trimethylamine. However, it seems that the positive experiences from the testing of antibiotics cannot be transferred to all chemical substances in general. It

seems useful to precheck the test substances in this respect or to control the microcalorimetric results by other techniques. The germ content or the optical density of the broth can be used for this purpose. Further investigations should be undertaken in this field.

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