Table 3. Effect of oxygen-free radical scavengers on the contraction-inhibition induced by the suffusate transferred from tissue bath A, in which EFS was applied to the aortic segment with intact EC.

Scavengers	n	% Contraction to 5-HT	
		Before treatment	After treatment
Superoxide dismutase (50 units/ml)	6	65.4 ± 4.8	99.0 ± 3.1 *
Catalase (150 units/ml)	6	62.3 ± 3.1	68.7 ± 3.9
Dimethyl sulfoxide (10 mM)	5	48.4 ± 5.2	63.9 ± 7.9
Mannitol (10 mM)	5	53.8 ± 3.8	67.7 ± 7.8
Gluthathione (36 μM)	5	69.3 ± 10.4	94.7 ± 7.1 *
Ascorbic acid (500 μM)	5	60.5 ± 4.9	89.7 ± 5.6 *

n, No. of experiments. * $p < 0.05$.

superoxide anion is involved in the breakdown of EDRF[15]. We have observed that EFS on the aortic segment with intact EC caused an inhibition of acetylcholine-induced cyclic GMP formation[6], which was prevented by prior treatment with SOD (data not shown). Consequently, both the existence of superoxide anion[15] in bath A and the two-min delay in suffusion of PSS[13, 14] to bath B, preclude the involvement of EDRF in the vascular smooth muscle relaxation.

Based on these considerations, it is presumed that this novel vascular-inhibitory factor is different from the well-known EDRF. However, it remains to be determined whether the ability of EC to release this vasodilator in response to superoxide anion can account for a diminished vascular reactivity in a variety of diseased states such as shock or ischemia-reperfusion injury.

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Flow microcalorimetry as a tool for an improved analysis of antibiotic activity: The different stages of chloramphenicol action

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Summary. Flow microcalorimetry in combination with photometric mass determination of staphylococci in suspension was used to reveal alterations in the intensity, extent and efficiency of bacterial metabolism during inhibition of protein synthesis by chloramphenicol. It could be demonstrated that these three parameters of metabolic activity were distinctly affected by this drug, and that the method described promises to be a more reliable tool for assaying the degree and the mode of bacteriostatic inhibition than the conventional determination of the minimum inhibitory concentration.
Key words. Microcalorimetry; antibiotics; minimal inhibitory concentration; photometry; staphylococci; metabolic activity; chloramphenicol.

The suitability of flow microcalorimetry for characterizing and quantifying antibiotic-induced alterations in the metabolism of microorganisms has been pointed out in several investigations[1-4]. Because living microorganisms produce heat, characteristic power-time curves (= pt-curves) could be recorded before and after addition of drugs to microbial suspensions[5]. Cultivation of bacteria in a conventional, complex nutrient broth yielded microcalorimetric curves which varied according to the intensity and the extent of the underlying processes, and which proved to be complex[6]; such curves have been considered to reflect a diauxic activity of the microbial

metabolism. For a quantitative microcalorimetric bioassay of antibiotics measurements of several parameters have been proposed: a) relative peak heights, b) time-to-peak-maximum, and c) time-to-return-to-baseline of heat production after addition of the drug [3]. We used a method combining the time-to-peak-maximum method with the peak height method. By relating the total heat production (representing the extent of metabolic processes) to the optical density (which served as mass indicator), we investigated the effects of the protein synthesis inhibition caused by chloramphenicol in staphylococci.

Materials and methods. Cells of *Staphylococcus aureus* strain SG 511 Berlin, from the culture collection of the Robert Koch Institute, Berlin-West, were grown at 37 °C in bactopeptone growth medium (Difco) pH 7.2 containing 2.5% bactopeptone and 0.5% NaCl. Chloramphenicol was obtained from Boehringer, FRG (lot 1479414). A fresh stock solution of the drug was prepared for each experiment and was added to the culture medium before the bacteria were inoculated. The minimum inhibitory concentration (MIC) of chloramphenicol (CAP) for the staphylococcal strain used was determined by a serial twofold dilution method. A solution of 1000 µg CAP/ml in growth medium was stepwise diluted 1:2 with medium down to 0.488 µg CAP/ml. The resulting solutions were inoculated with equal numbers of staphylococci (final concentration: 10^6/ml) and incubated at 37 °C for 20 h. The lowest drug dose leading to a visually discernible absence of turbidity was then termed the MIC.

Calorimetry. Stationary phase bacteria from an overnight culture obtained by shaking staphylococci at 37 °C in the growth medium served as the inoculum for the calorimetric experiments which were performed in the same nutrient medium. The culture was shaken for 10 h at 37 °C, starting from an initial cell concentration of 10^8/ml as determined by light microscopy, which corresponded to an optical density of 0.1 at 578 nm.

During that whole period of time, an aliquot of the bacterial suspension was pumped through the measuring coil (0.7 ml) of an LKB 2107 flow microcalorimeter at a constant flow rate of 40 ml/h and returned to the culture flask. A stable baseline was obtained by pumping the pure growth medium through the device for about 1 h before inoculation of the staphylococci. Integration of the recorded pt-curves was done using an electronic integrator (Digikon, Kontron).

Spectrophotometry. Photometric measurements were performed continuously at 578 nm using a Zeiss PM 6 flow-through spectrophotometer. To ensure the highest possible accuracy of the final optical density readings, tenfold dilutions of the suspensions were additionally measured or the readings were corrected by comparison with a standard curve.

Results. The MIC of CAP for *S. aureus* SG 511 Berlin proved to be 15.6 µg/ml. The effect of CAP on the heat production of staphylococci is shown in figure 1 A. Untreated cells generated a characteristic heat-production

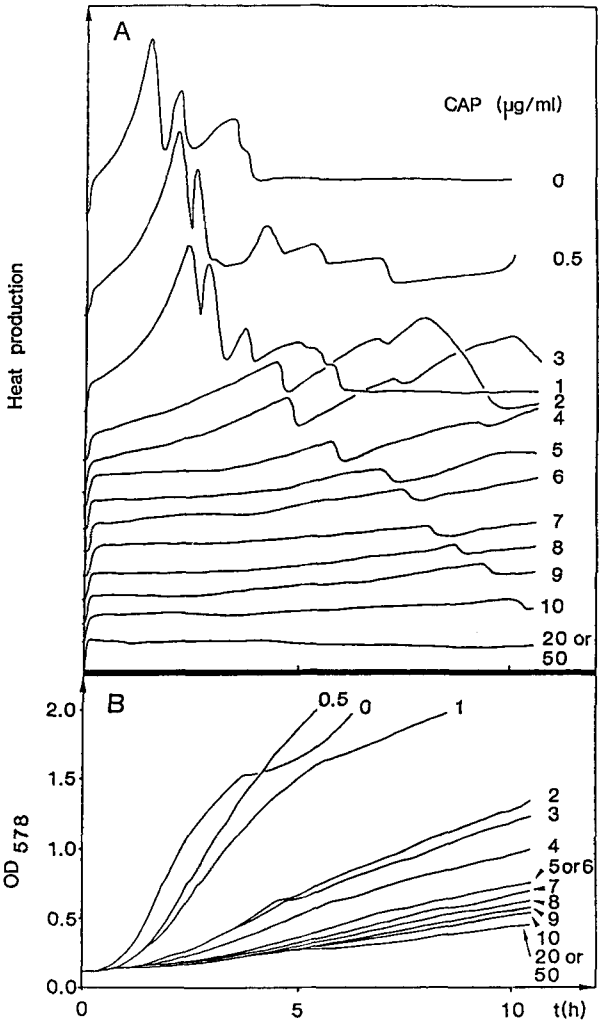

Figure 1. Effect of different doses of CAP on heat production (arbitrary units, panel A) and on optical density (panel B) of *S. aureus* SG 511 Berlin growing in bactopeptone broth. The heat production curves have separate origins on the Y axis and are drawn one beneath the other, to improve the clarity of the illustration. The antibiotic was added at t = 0 just before inoculation of the staphylococci.

curve with three peaks during the first 5 h of the experiment. Just as in an earlier investigation [5], an exponential development of the turbidity of the staphylococcal suspension could be demonstrated for this period of peak occurrence (see fig. 1 B). After that, a constant low level heat output was recorded. When the reproducibility of the results with the control culture was scrutinized in 6 independent experimental runs, the standard deviations were ±10% for the integration values of the heat curves, ±4% for the first peak height and ±3% for the temporal appearance of this first peak, respectively.

With increasing concentrations of the antibiotic (i.e., from 0.5 to 10 µg/ml), a linear increase of the time of appearance of the first peak maximum was observed (compare 'time' in fig. 2), until, in the presence of 20 µg/ml CAP or more, no heat peak could be detected any longer and the pt-curves were parallel with the baseline

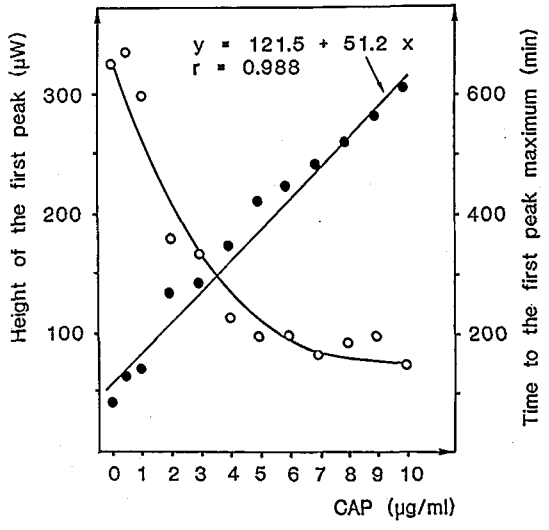

Figure 2. Effect of different doses of CAP on height (=o-o) and time-to-maximum (=●—●) of the first peak in pt-curves of *S. aureus* SG 511 Berlin growing in bactopeptone broth.

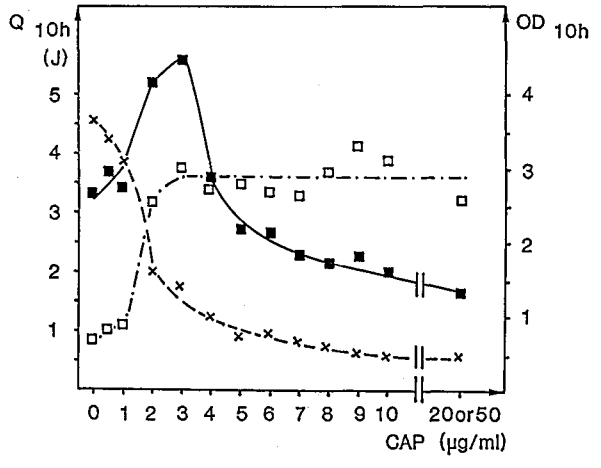

Figure 3. Effect of different doses of CAP on total heat production Q = ■—■) and on the final optical densities (OD = x—x) of *S. aureus* SG 511 Berlin after a 10-h cultivation. The initial optical densities were 0.1 corresponding to 10^8 cells/ml. Specific heat production was calculated as the ratio of total heat liberated within 10 h (i.e., the areas under the pt-curves in fig. 1 A) and final optical density (Q/OD = □ · — · □).

(fig. 1 A). During the 10 h of observation, the absolute height of the first peak already decreased to a minimal peak height at about 5 µg/ml CAP, apparently obeying an exponential function (compare 'height' in fig. 2). Astonishingly, the total amount of heat liberated, as represented by the total areas under the curves in figure 1 A, increased at first in the concentration range of 1 to 3 µg/ml CAP and decreased again thereafter (Q in fig. 3).

The effect of CAP on bacterial growth rate, as seen by the development of the optical densities of the suspensions, showed slight effects at 0.5 and 1 µg/ml CAP and a substantial reduction with larger doses (fig. 1 B).

The final corrected optical densities of the CAP-treated cultures after 10 h of cultivation (OD 10 h) were reduced in the presence of even low CAP doses, as compared to untreated control cells, but the lowest OD level reached with high CAP doses was still five times as high as the initial OD of 0.1 (compare OD in fig. 3). The specific total heat production, calculated as the ratio of the total area under the pt-curve (see fig. 1 A), and the corresponding final optical density, reached a constant level beyond 2 µg/ml CAP (Q/OD in fig. 3) which was more than three times as high as the corresponding ratio for untreated bacteria.

Discussion. The continuous monitoring of catabolic processes via microcalorimetry yielded considerably more information about the antibacterial activity of CAP on staphylococci than did a conventional MIC assay. Even different inhibition kinetics could be detected, revealing some new characteristics of antibiotic action. Whereas the time of a heat maximum proved to be directly proportional to increasing drug concentration, its absolute height, reflecting the intensity of the causative process[6], already decreased to a minimum at low drug doses (fig. 2). Thus, with the microcalorimetric method, it was possible to record and to distinguish between a CAP-induced retardation and CAP-induced reduction of certain metabolic events.

Remarkably, even at low, sub-MIC doses of CAP, the ratio between total heat output and final optical density indicated a loss of bacterial metabolic capability for synthesizing cellular components, since the heat quantity released per cell mass was increased (Q/OD in fig. 3). This result points to an initial inhibition of anabolic processes when protein synthesis begins to be affected by CAP. A comparable decrease in the yield of biomass, accompanied by an increased energy loss in form of heat and CO_2, has earlier been shown to take place during Nalidixin-treatment of *Klebsiella*[8], suggesting that this process may generally be induced by impairing biosynthesis at the chromosomal or ribosomal level. Since the anabolic efficiency and the intensity of heat production (the latter being an indicator of the catabolic activity of the microorganisms[9]) were strongly affected already at CAP concentrations below the MIC (i.e., up to 2 respectively 5 µg/ml), one has to consider the possibility that the MIC measured visually (15.6 µg/ml) did not reflect the true minimum inhibitory drug concentration for the investigated staphylococci. Rather, as the microcalorimetric data signalized, even a CAP dose of as little as about 5 µg/ml must be considered to be inhibitory with our strain.

Since it was possible, using microcalorimetry, to detect important metabolic consequences in staphylococci even at very low CAP concentrations, we concluded that this technique may be generally suitable for examining the effects of subinhibitory concentrations of any antimicrobial agents.

It should be noted that even at the highest CAP concentration tested (50 µg/ml), the final optical density of the suspension after 10 h of cultivation was still five times as

high as the initial one (fig. 3). While, in general, any increase in optical density of a bacterial culture reflects an increase in the number of cells, such an increase occurs with gram-positive bacteria growing in the presence of CAP on different grounds. It is well known that wall synthesis in gram-positive microorganisms is not suppressed when protein synthesis is blocked [10], and, more recently, we could demonstrate by combined electron microscopic and spectrophotometric studies that the increase in optical density measured with CAP-treated staphylococcal cultures was not due to an increased number of cells but to the drug-induced growth of thickened cell walls. Obviously this phenomenon may also influence the MIC determination, which depends on optical properties of the bacterial suspension, as well. However, since at present the only MIC data examined are for our SG 511-strain (and two other staphylococcal strains with basically the same CAP sensibility), further investigations are required to prove the correctness of this assumption for staphylococcal strains with lower MICs for CAP also.

Since cell wall thickening can be induced by other bacteriostatic antibiotics, too (i.e., tetracyclines [11] or macrolides [12]), the possibility of false data must be taken into account when conventional MIC tests are performed with these drugs. Altered MIC definitions, considering a certain increase in cell number or in colony-forming units (CFU test), may deliver more realistic results but are laborious and slow as compared to microcalorimetry; moreover, it is only possible to obtain a single result at the end of an experiment, whereas with flow microcalorimetry inhibition effects are recorded continuously. Therefore, particularly when developing new chemotherapeutic agents or for clinical studies of bacterial susceptibilities, the microcalorimetric assay of the reduction, delay and loss of efficiency of microbial metabolism may yield better evaluation criteria for the antibiotic activity of a drug than do MIC techniques or photometric methods alone.

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Fungitoxicity of *m*-fluorophenylalanine-containing peptides towards *Pythium ultimum*

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Summary. The tripeptide L-*m*-fluorophenylalanyl-L-alanyl-L-alanine was much more fungitoxic towards *Pythium ultimum* than the dipeptide L-*m*-fluorophenylalanyl-L-alanine or *m*-fluorophenylalanine. The fungitoxicity of the tripeptide was reduced by L-alanyl peptides and phenylalanine, but not by other amino acids. In contrast, the fungitoxicity of *m*-fluorophenylalanine was unaffected by peptides, and was antagonized by several amino acids. These results suggest the effective delivery of *m*-fluorophenylalanine into the cell by a tripeptide carrier.
Key words. Amino acid transport; fluorophenylalanine; peptide transport; *Pythium ultimum.*

Peptide permeases generally exhibit less specific substrate range than amino acid permeases [1]. This characteristic can be exploited to facilitate entry of impermeant amino acid analogs, or other molecules, into the cell by delivery in the form of a peptide which may then undergo intracellular hydrolysis. In various natural [2–5] and synthetic [1, 6–9] antimicrobial peptides this concept of 'illicit transport' is utilized to enable a toxophore to reach its intracellular site of action. In *Candida albicans* [10], for example, fungitoxicity of the amino acid toxophore *m*-fluorophenylalanine (*m*-FPhe) is greatly enhanced by delivery as the di- or tripeptides, L-*m*-fluorophenylalanyl-L-alanine (L-*m*-FPhe-L-Ala) and L-*m*-fluorophenylalanyl-L-alanyl-L-alanine (L-*m*-FPhe-L-Ala-L-Ala).

Information about the existence and properties of peptide permeases in plant pathogenic fungi would be helpful in the design of agricultural fungicides which could be taken up by peptide transport. Unfortunately, the limited evidence for such permeases derives from studies of polyoxins, which are transported by peptide permeases in