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MICROCALORIMETRIC ASSESSMENT OF GYRASE-INHIBITORS

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SUMMARY

The microcalorimetric procedure gives results that, with respect to MIC and MBC, correlate well with those obtained by traditional methods. From the shape of the power-time-curves for E. coli ATCC 25922, it is possible to distinguish "old gyrase-inhibitors" from "fluoroquinolones". Furthermore, at concentrations producing a maximal bactericidal effect nalidixic acid differs microcalorimetrically from cinoxacin and pipemidic acid. At subinhibitory concentrations all gyrase-inhibitors exhibit a uniforn double-peaked power-time-curve, a fact that can be used for identifying substances belonging to this group.

INTRODUCTION

In 1962, with the administration of nalidixic acid, a so-called gyrase inhibitor was employed for the first time in antimicrobial chemotherapy. The numerous new, fluorinated and much more active derivates of nalidixic acid that have been developed in recent years are chemically totally unrelated to other chemotherapeutic agents.

On the basis of their antimicrobial activity a distinction is made between "old gyrase inhibitors" and "new gyrase inhibitors". In addition, the substances at present available can be classified according to their chemical structure as follows: 1. naphthyridine (nalidixic acid, enoxacin), 2. cinnoline (cinoxacin), 3. pyridopyrimidine (pipemidic acid) and 4. quinoline (norfloxacin, ciprofloxacin, ofloxacin, fleroxacin) (Fig.1).

Until recently, the high activity of modern gyrase inhibitors was generally believed to be connected with the basic structure of norfloxacin. It is now known, however, that fluorination and the piperazine ring of the side chain are not indispensible for high antibacterial activity. As a result, a large number of possible substances have now to be tested for antibacterial activity. The advantages of the gyrase inhibitors, such as their rapid bactericidal effect, broad activity spectrum, suitability for oral administration, low degree of breakdown, and low tendency to confer resistance have set off an intensive search for new derivates.

The aim of our investigations was to determine to what extent the power-time-curves obtained by microcalorimetry constitute a suitable criterion

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for evaluating and classifying newly developed substances belonging to this group of compounds, of which it can be said with certainty that, at the molecular level, they attack at one and the same point.

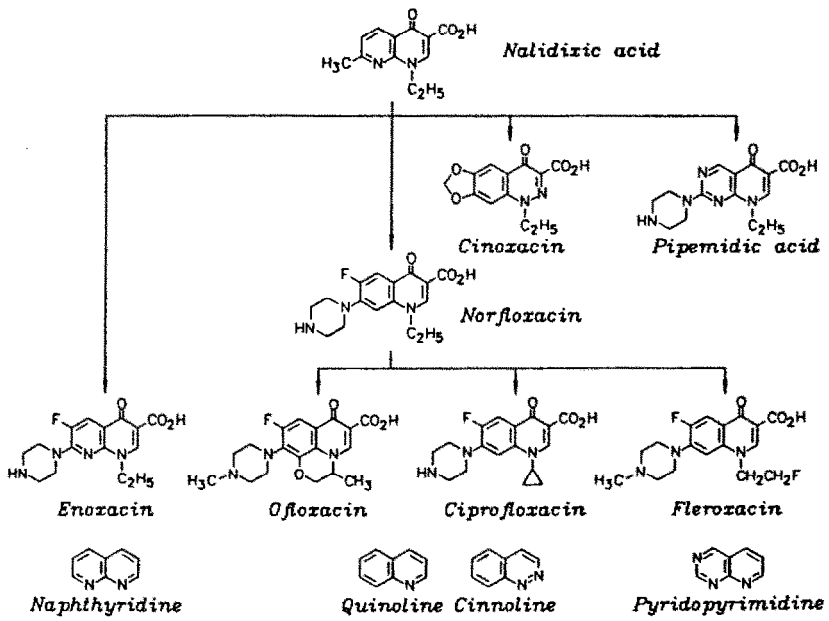

Fig. 1. Gyrase-inhibitors at present available.

MATERIALS AND METHODS

Microcalorimeter

We used a LKB calorimeter 2107/210 (Flow System). The heat produced by the bacteria is transformed into thermal currents by a semiconductor situated in a measuring cell with a capacity of 0.7 ml. Our apparatus had been adjusted to permit measurements over the range of 0-30 µV.

Recorder

LKB 2210 2-Channel Potentiometric Recorder with a running speed of 0.5 mm/min.

Test organism

Escherichia coli ATCC 25922, a commercially available lyophilized standard laboratory control strain, was used throughout this study.

Medium

Columbia broth (GIBCO), a high quality all-purpose medium (pH 7.5), was used.

Antimicrobial agents

Antimicrobial stock solutions were prepared from standard powders, using the following agents: nalidixic acid (Sigma, St. Louis, USA), cinoxacin (Eli Lilly, Indianapolis, USA), pipemidic acid (Madaus, Cologne, FRG), norfloxacin (Merk Sharp & Dohme, Haarlem, USA), ofloxacin (Hoechst Austria, Vienna, A), ciprofloxacin (Bayer, Leverkusen, FRG), enoxacin (Parke, Davis & Company, Freiburg, FRG), fleroxacin (Hoffmann - La Roche, Basel, CH), and novobiocin (Sigma, St. Louis, USA).

Experimental Method

In order to demonstrate the microcalorimetric pattern produced by E. coli ATCC 25922, 5 ml of bacterial suspension (grown from a single colony incubated for 12 h in 5 ml broth) were inoculated into 500 ml of broth, yielding an initial density of 10^6 bacteria/ml. The broth was then stirred continuously with a magnetic stirrer during incubation in a waterbath at 37°C. After passage through the microcalorimeter at a rate of 25 ml/h the broth was collected in a separate container. Thermal power was recorded as a power-time-curve (p-t-curve).

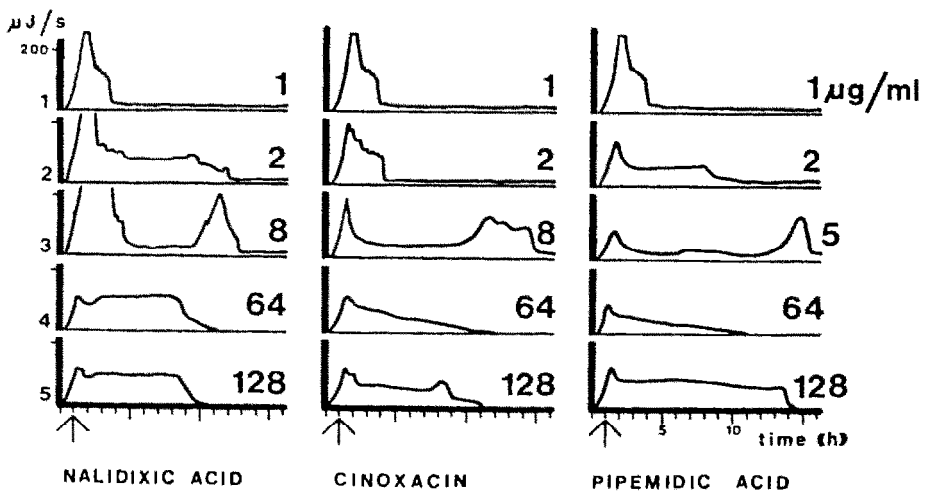

Fig. 2. Power-time-curves of E. coli ATCC 25922 with varying concentrations of nalidixic acid, cinoxacin, and pipemidic acid.

RESULTS

Nalidixic acid

The power-time-curves of E. coli ATCC 25922 varied depending on the amount of nalidixic acid added, the antimicrobial agent being added in each case 60 min after beginning of the experiment (Fig. 2). At a concentration of 1 µg/ml, nalidixic acid did not affect the metabolism of the bacteria, as measured by microcalorimetry. As in the curve obtained for E. coli in the absence of antibiotics, a peak was attained approximately 2 h from the start, followed by a steep, smooth drop, interrupted for an interval of about 90 min during wich the thermal decline slowed down, after which the steep drop to the baseline was resumed. Nalidixic acid at a concentration of 2 µg/ml caused an increase in thermal output beyond the range of measurement, followed by a fall to a plateau at 80 µJ/s and a return to the base-line, which was reached about 12 h from the start. A concentration of 8 µg/ml led to a double-peaked curve, the peaks in the p-t-curve being separated for over 5 h by a plateau at 30 µJ/s. Nalidixic acid at concentrations of 64 µg/ml and 128 µg/ml halted the expected further increase in thermal output and led to plateau formation at a level of 100 µJ/s and a fall to the zero-line 10 h from the start. An identical p-t-curve was found after addition of 1000 µg/ml (p-t-curve not presented).

Cinoxacin and Pipemidic acid

Neither cinoxacin nor pipemidic acid in concentrations of up to 1 µg/ml had any effect on the thermal output of the bacteria (Fig. 2), whereas both agents in concentrations of 2 µg/ml increased the duration of the plateau-like interval interrupting the fall from maximal thermal activity to the baseline. Increasing the concentration further (cinoxacin 8 µg/ml, pipemidic acid 5 µg/ml), led to a double-peaked curve; contrary to the effect of nalidixic acid, the maximal thermal output was less than 200 µJ/s. The effects of both cinoxacin and pipemidic acid at concentrations of 128 µg/ml and 1000 µg/ml (latter p-t-curve not presented) were similar to those caused by nalidixic acid. At concentrations of 64 µg/ml the p-t-curves for cinoxacin and pipemidic differed from the curve obtained after addition of nalidixic acid; after an abrupt fall of 30 µg/ml a continuous decline set in and the zero-line was reached 10 h after addition of the agents.

Norfloxacin, Ofloxacin, Ciprofloxacin, Fleroxacin, Enoxacin

Fig. 3 shows the p-t-curves of E. coli ATCC 25922 under the influence of norfloxacin, ofloxacin and ciprofloxacin in varying doses. Effects of fleroxacin and enoxacin on E. coli are presented in Fig. 4. In contrast to the older related compounds such as nalidixic acid, cinoxacin and pipemidic acid, addition of high doses, i.e. 128 µg/ml and 1000 µg/ml (latter p-t-curves not presented) did not lead to a plateau in the p-t-curve. Norfloxacin, ofloxacin and fleroxacin at a concentration of 1 µg/ml (ciprofloxacin at 0.1 µg/ml and

enoxacin at 2 µg/ml) produced only a single peak in the respective p-t-curve, which then gradually declined, reaching the zero line 7 h after addition of the agent. At a concentration of 0.006 µg/ml, however, each of the former three agents caused a double-peaked p-t-curve (as did ciprofloxacin at 0.01 µg/ml and enoxacin at 0.2 µg/ml), similar in pattern to that produced by 8 µg/ml cinoxacin and 5 µg/ml pipemidic acid. These curves showed a greater spread since the first peak was followed by a second slight increase in thermal activity; the return to the baseline 7 to 10 h delayed, as compared with the behaviour in the absence of antimicrobial agents. After addition of enoxacin (0.2 µg/ml) this drop to the baseline was delayed by 2 h, and after addition of ciprofloxacin (0.01 µg/ml) by 4 hours.

Novobiocin

Unlike the above substances, novobiocin (which is not a so-called gyrase inhibitor) completely failed to eliminate thermal activity. Gyrase inhibitors attack at the A subunit of the E. coli DNA gyrase, whereas the antibiotic novobiocin exerts its effect via inhibition of the gyrase-B subunit. Novobiocin at a concentration of 1 µg/ml had no influence on the heat-production of E. coli growing in Columbia Broth (Fig.3). Increasing the dose led to plateau formation in the p-t-curve; 20 µg/ml novobiocin halted a further increase in thermal

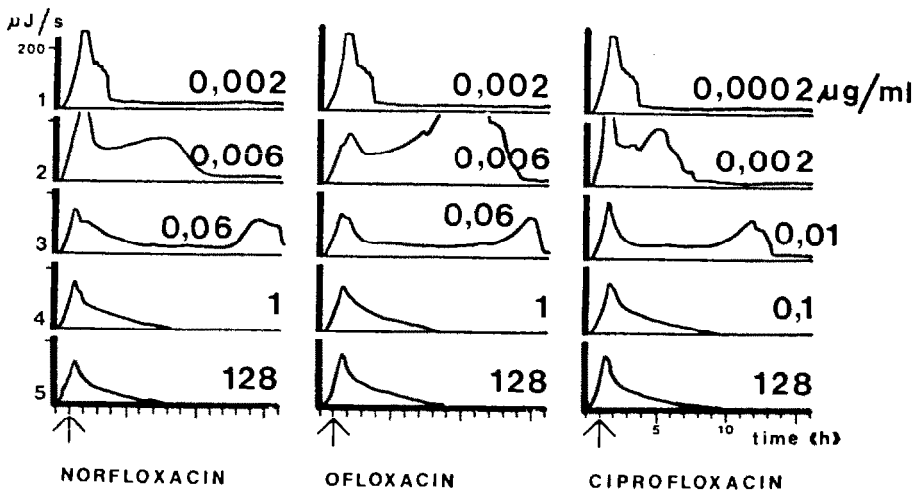

Fig. 3. Power-time-curves of E. coli ATCC 25922 with varying concentrations of norfloxacin, ofloxacin, and ciprofloxacin.

output and led to the formation of a plateau which dropped to the base-line 14 h after the addition of the substance. Under the influence of 128 µg/ml and 1000 µg/ml (latter p-t-curve not presented) the plateaus in the curve persisted until 7 h after addition of the antimicrobial agent.

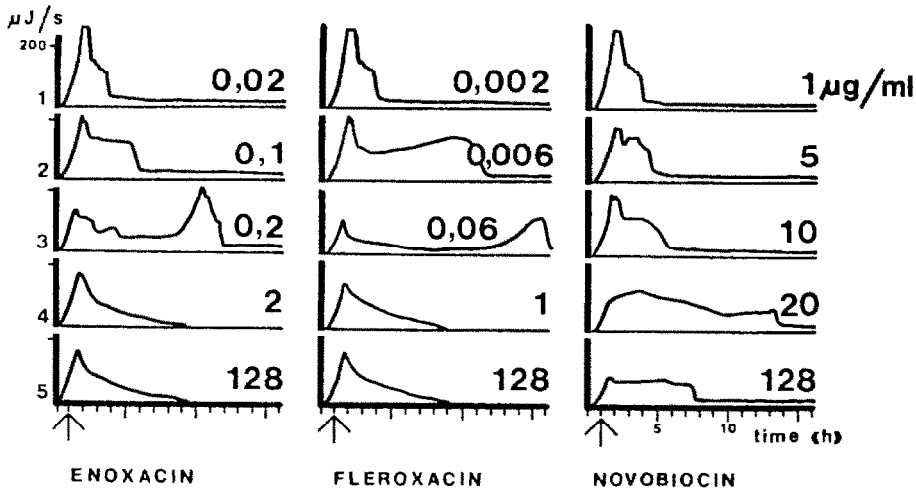

Fig. 4. Power-time-curves of E. coli ATCC 25922 with varying concentrations of enoxacin, fleroxacin, and novobiocin.

DISCUSSION

DNA-gyrase was discovered in 1976 in the course of studies on the mechanism of action of nalidixic acid (1). Today, a variety of pyridone-carboxylic acid antimicrobial agents are available, comprissing older related compounds such as nalidixic acid and new fluorinated compounds. Most of our knowledge regarding the effects of these agents has come from studies of E. coli and E. coli DNA gyrase. E.coli DNA gyrase has been shown to be a tetrameric complex composed of two 100.000 dalton A subunits and two 90.000 dalton B subunits. The enzyme gyrase catalyses an overspiralization of bacterial DNA by drawing a double chain through a temporary enzymatically produced gap in the DNA. Quinolones prevent the enzymatic repair of the ruptured DNA and thus bring about a DNA-gyrase complex which can cause breaks in the double chain. The mapping and sequencing of these gyrase breaks in the DNA in-vivo and in-vitro revealed that the different quinolones are blocked at the same position in the DNA. The exact

mechanism underlying their bactericidal effect is poorly understood, and a satisfactory explanation for the differences in antimicrobial activity has not yet been found.

The technique of microcalorimetry allows uninterrupted observation of the effects of antimicrobial agents on bacterial metabolism. In contrast to the traditional methods, microcalorimetry assesses the thermal activity of bacteria rather than their multiplication. For the commercially available agents tested, this procedure offers a rapid, simple and reliable means of placing the substance concerned within the scheme at present used for classifying gyrase inhibitors.

In assessing the MIC/MBC values (minimal inhibitory concentration/minimal bactericidal concentration) it should be noted that in our microcalorimetric protocol the cell concentration at the time of addition of the antimicrobial agent is 10^7 CFU/ml, as compared to 10^4-10^5 CFU/ml in the usual test procedures (ref. 2). The high initial number of cells explains the apparently high initial MIC values of the old gyrase inhibitors. At a nalidixic acid concentration of 64 µg/ml the pattern of the p-t-curve showed a typical bactericidal effect i.e. extinction of the thermal activity. RONALD gives a value of 12 µg/ml for the MIC of E. coli starting with 10^7 CFU/ml (ref. 3). This is in good correlation with our microcalorimetric results, where the addition of nalidixic acid in concentrations below 12 µg/ml failed to halt a further increase in thermal output.

In general, a bactericidal effect can be achieved with gyrase inhibitors at concentrations equal to or double the MIC (ref. 4).

Nalidixic acid is one of a series 1,8 naphthyridine derivates first described by LESHER and colleagues in 1962 (ref. 5). It has been commercially available as a chemotherapeutic agent since 1964/65. Cinoxacin is a synthetic organic acid with a cinnoline as the basic ring structure, is chemically related to nalidixic acid and was developed by WICK et al. in 1973 (ref. 6). Further, its antimicrobial spectrum is similar to that found for nalidixic acid. MARDH et al. found an MBC of 12.o µg/ml for E. coli (using an inoculum of 10^7 CFU/ml) (ref. 7). This is in good agreement with our microcalorimetric results which shows that a rise in thermal power could not be prevented by concentrations below 12 µg/ml. The antibacterial in-vitro activity of pipemidic acid, a substance developed in 1975 by SHIMIZU et al., was investigated by PETERS et al. who found that the MIC values for E. coli were only about half those obtained with nalidixic acid (refs. 8-9). This agrees well with our microcalorimetric results, which showed a bactericidal action of pipemidic acid at concentrations above 5 µg/ml.

Unlike the old gyrase inhibitors the MIC/MBC values for the fluoroquinolones are not significantly influenced by the inoculum size. Only a few authors, such

as DRIVER in the case of ciprofloxacin (ref. 10), have reported different results, i.e. a drastic increase in MIC as the inoculum was increased. Using the microcalorimetric technique, an influence of the initial cell number could not be detected for fluoroquinolones.

Norfloxacin, described in 1980 by ITO et al. (ref. 11), was the first fluorine-containing quinolone derivate. According to HOHL, the MIC/MBC values for E. coli ATCC 25922 are 0.12/0.12 µg/ml and the MIC remains constant even when the inoculum is increased from 10^3 to 10^6 (ref. 12). This agrees well with our microcalorimetric results which showed 0.06 µg/ml to be the highest concentration of norfloxacin without a bactericidal effect.

Ofloxacin, another new 4-quinolone derivate now available as a therapeutic agent (ref. 13), has, according to ROTTER and HIRSCHL, an MIC and an MBK for E. coli of 0.12 µg/ml (ref. 14). The differences in MIC/MBC due to differences in the size of inoculum (10^4 and 10^6 CFU/ml) were considered by BAUERNFEIND et al. to be "minimal" (ref. 15). Here, too, the values obtained by measuring thermal power correlate well with those obtained by traditional methods.

Ciprofloxacin is another new quinolone derivate resembling nalidixic acid, but with a distinctly stronger antibacterial effect (ref. 16). According to ZINNER and DUDLEY the MIC/MBC of E. coli ATCC 25922 is 0.016/0.016 (ref. 17). INOUE reports that the antibacterial action of Ciprofloxacin, too, is only slightly affected by inoculum size (ref. 18). For ciprofloxacin 0.01 µg/ml was the highest concentration that failed to give a bactericidal pattern in the p-t-curve, which once more means agreement of the microcalorimetric results with those obtained by other authors using the traditional serial broth dilution test.

Enoxacin is a 4-quinolone developed in 1980 by MATSUMOTO (ref. 19). Testing the in-vitro activity of enoxacin on 25 strains of E. coli REEVES found for E. coli an MIC of 0.25 µg/ml; the MBC determinations performed at the same time gave results either identical to or double those for MIC (ref. 20). According to BAUERNFEIND and ULLMANN there are only minimal differences between MIC for 10^4 and 10^6 CFU/ml (ref. 15). In the microcalorimetric tests the highest concentration of enoxacin that was not capable of stopping a further rise in thermal activity was 0.2 µg/ml. Concentrations that were found to be bactericidal by means of the standard dilution test also effected a decline in thermal power, the p-t-curve dropping to the zero-line.

Fleroxacin, the youngest representative of the class of registered fluoroquinolones, unlike the other known quinolones, possesses three fluorine atoms on the quinolone ring system. In this case, too, the size of the inoculum has very little effect on the activity of the agent (ref. 21). The MIC for E. coli is 0.13 µg/ml according to BAUERNFEIND (ref. 22), and the MBC/MIC ratio is reported by Hohl to be 1 (ref. 23). Similar results were obtained in our

microcalorimetric procedure, in which thermal activity was extinguished at fleroxacin concentrations above 0.06 µg/ml.

Quinolones form a tight complex with gyrase on DNA and by interfering with DNA breakage and rejoining inhibit all topoisomerase activities of the enzyme. In contrast, a second unrelated class of gyrase inhibitors that includes novobiocin and coumermycin A_1 exhibits a quite different inhibitory mechanism. Coumermycin and novobiocin are not related chemically to the 4-quinolone antibacterials. They block access of ATP to the gyrase B subunits and thus inhibit the ATP-requiring activities of gyrase (refs. 24-25). Novobiocin was discovered in 1955 in culture filtrates from Streptomyces spheroides and belongs to no particular group of antibiotics. The inhibition values (MIC) are considerably influenced by the size of the inoculum and by the pH value; according to OTTEN the MIC is about 25 µg/ml for E. coli (ref. 4). The microcalorimetric curve for novobiocin differs markedly from the p-t-curves of the so-called gyrase inhibitors. In contrast to the latter, thermal activity is still detectable following the addition of higher concentrations, which in the microcalorimetric method indicates bacteriostatic effect. Also in contrast to the gyrase inhibitors, at no concentration range does novobiocin give a double-peaked curve. The double-peak pattern of the p-t-curves at concentrations just below the MIC seems to be a significant characterisitc of gyrase inhibitors. In no other group of substances have we so far seen a similar picture.

With regard to MIC and MBC it can be said that the microcalorimetric results correlate with those of traditional test methods. For quinolones the MBC is not identical with the concentration with maximal bactericidal effect: the bactericidal effect of the drug increases with increasing concentration. The greatest bactericidal effect is observed at 90 µg/ml nalidixic acid, 90 µg/ml cinoxacin and 50 µg/ml pipemidic acid respectively (ref. 26). The old gyrase inhibitors are clearly inferior to the newer substances with respect to speed of killing (ref. 16), a fact which is also confirmed by our microcalorimetric studies. Following the addition of 64 µg/ml nalidixic acid, cinoxacin and pipemidic acid, 10 h elapse before thermal activity is extinguished, whereas for the new gyrase inhibitors the corresponding time interval is only 7 h (in the concentration range of maximal bactericidal activity). SMITH reports the maximum bactericidal effect for norfloxacin to be 1.5 µg/ml, for ofloxacin 0.9 µg/ml and for ciprofloxacin 0.15 µg/ml (ref. 26); for enoxacin this value is 4 µg/ml and for fleroxacin 1 µg/ml (authors' own unpublished data).

The microcalorimetric method reveals that nalidixic acid does not fit into either of the traditional categories of old and new gyrase inhibitors but forms a class of its own. Unlike cinoxacin and pipemidic acid the curve for 64 µg/ml nalidixic acid does not indicate a continous falling off in thermal activity but

remains almost constant for the first 8 h following addition of the agents. This could imply that, as well as differing in their activity per unit weight, the old quinolones also differ with respect to the nature or their antibacterial effect. At high concentrations the microcalorimetric behaviour of the old gyrase inhibitors differs uniformly from that of the new gyrase inhibitors, the p-t-curves of the latter showing a continuous decrease in thermal activity following addition of the active substance.

With increasing concentration above the maximal bactericidal dose a so-called paradoxical reaction occurs. This paradoxical response in which higher doses of quinolone result in less killing, is probably due to secondary inhibition of RNA synthesis and protein synthesis by the drug (ref. 27). Employing microcalorimetry the effect can be demonstrated most clearly for cinoxacin and pipemidic acid: by increasing the doses to 128 and 1000 µg/ml the form of the p-t-curves obtained corresponds to that of nalidixic acid, and in the case of pipemidic acid even results in a prolongation of the registrable thermal activity. Using traditional methods, the only evidence obtainable for this paradoxical reaction is the different degree of bacterial killing. By means of microcalorimetry we were also able to detect an apparently similar phenomenon in novobiocin. Novobiocin, bacteriostatic to E. coli, had a much more pronounced effect on the shape of the curve at a concentration of 20 µg/ml than at concentrations of 128 or 1000 µg/ml. Even if, at the molecular level, the point of attack differs, the paradoxical reaction could be a property of all gyrase-inhibiting substances.

Summarizing, it can be said that the results obtained by means of the microcalorimetric procedure are in good correlation with those obtained by traditional methods. The microcalorimetric procedure should prove useful in evaluating and classifying newly developed compounds of the quinolone group. Furthermore, the good correlation between microcalorimetric results and those obtained by other methods emphasizes the value of this procedure in those fields of application where traditional methods are connected with obstacles (refs. 28-29).

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