INHIBITION OF R-FACTOR TRANSFER BY LEVALLORPHAN

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Received July 30, 1971

SUMMARY - The transfer of an R-factor from Proteus Rettgeri to Escherichia coli W 677 was strongly inhibited by levallorphan at concentrations which do not affect growth of either the donor or the recipient cells. The effect is not due to the transfer of the episom itself but to inhibition of mating-pair formation.

INTRODUCTION - At least two consecutive steps can be distinguished in the transfer of R-factors: (i) the formation of mating-pairs and (ii) the transfer of the episome from the donor to the receptor cells. According to Novotny, Knight and Brinton (1) conjugation can be inhibited by male specific phages and Knolle (2) has reported that, inversely, infection of the phage can be inhibited by conjugation. We were able to show that levallorphan, a N-allyl-derivative of levorphanol, effects an inhibition of infection by male specific phages and even a desorption of already adsorbed phages from the sex-pili (3). Our suggestion that levallorphan might affect the membrane or the membraneous extensions the pili of the bacterial cells would gain further support if levallorphan could be shown to inhibit some step in the transfer of R-factors. In the present study it is shown that at least most of the inhibition of transfer of R-factors by levallorphan is due to the prevention of formation of mating-pairs, or their cleavage.

MATERIALS & METHODS - Bacterial strains: As donor a Proteus Rettgeri strain, isolated from clinical investigation samples, was used and an Escherichia coli K12, W 677 - R^-, F^- served as receptor strain. In Table 1 the resistance patterns are listed.

Medium: Bacto Antibiotic Medium 3 (Bacto-Penassay broth) was used for mating, growing and dilution of the bacteria. The pH was adjusted to 8.0 with triethanolamine. Plating was carried out on this medium supplemented with 1.5 % Bacto agar and the antibiotics in concentrations indicated in

Tab. 1. This agar was usually designated as "antibiotics agar", if not otherwise indicated. The antibiotics were sterilized by membrane-filtration and added to the agar at 50°C before pouring of plates.

TABLE 1

Antibiotics	Concentration	Recipient E.coli W 677 Growth	Donor Proteus Rettgeri Growth	Transferable Resistance of Donor
Streptomycin	500 µg/ml	+	-	?
Tetracyclin	40 µg/ml	-	+	-
Chloramphenicol	20 µg/ml	-	+	+
Kanamycin	70 µg/ml	-	+	+
Neomycin	100 µg/ml	-	+	+

Mating method: Bacteria were grown overnight at 37°C in broth to stationary phase. As transfer to fresh medium did not give higher yields, the stationary cultures of Proteus and E.coli were directly mixed together in equal amounts (1 ml + 1 ml). In the stationary phase cultures E.coli usually had a cell density of 5 x 10^8 cells/ml and Proteus Rettgeri one of 6 x 10^8 cells/ml.

The cell mixtures were incubated at 37°C without shaking. Samples of 30 µl were taken at different time intervals and plated on antibiotics agar. After about 16 hours of incubation of the plates the resistant colonies were counted with help of a colony-counter.

Measurement of mating-pairs: For measurement of mating-pairs a dilution technique similar to that of Curtis III and Stallions (4) was applied. The high cell density culture mixtures (20 µl) were diluted 1:500 into fresh Penassay broth and the diluted mixtures were allowed to stand at room temperature for 60 minutes. Then the tubes were centrifuged at 15 000 RPM for 10 minutes at 25°C. The sediment was suspended in 3 ml of warm (50°C) antibiotics agar which contained only 0.8 % agar. This suspension was poured onto the surface of the normal antibiotics agar plate. The plates were incubated at 37°C overnight and the colonies were counted. The number of colonies was taken as a measure of the number of mating-pairs in the mating mixture at the time of dilution.

Estimation of cells which have lost the R-factor: For estimation of donor and recipient cells which spontaneously and in presence of levallorpha have become sensitive to the applied antibiotics a penicillin screening technique similar to that of Watanabe and Fukasawa (5) was used. Overnight cultures (5 ml) of the bacteria were grown in the presence (0.7 mM) and

absence of levallorphan. The cell density was estimated by a Thoma counting chamber. The bacteria were then centrifuged in the cold and washed three times with cold, sterile saline. The sediment was resuspended in 10 ml of sterile saline and 50 µl amounts added to 5 ml Penassay broth containing 200 units/ml penicillin in addition to (i), 70 µg/ml kanamycin (ii), 70 µg/ml kanamycin and 100 µg/ml neomycin and (iii) 70 µg/ml kanamycin, 100 µg/ml neomycin and 20 µg/ml chloramphenicol. The bacteria were incubated overnight at 37°C. The tubes were then centrifuged and the sediment suspended in 1 ml of sterile saline. Portions of 50 µl were plated on agar plates without antibiotics and, in one case, with the various antibiotics (as in broth) to check the sensitivity of the survivors. The colonies of the surviving bacteria were counted. The E.coli culture had been previously made resistant by the transfer of the R-factor from Proteus.

Transfer kinetics: Transfer kinetics were carried out by interrupted mating experiments (6). After mixing the E.coli and the Proteus overnight culture samples were taken from the mixture at different time intervals. The samples were vigorously shaken on a Cenco whirlmix for 40 sec. and portions of 40 µl were plated on selective agars containing first, each antibiotic separately, and then after the sequence of transfer has been established, combinations as indicated.

RESULTS - a) Inhibition of the transfer of R-factors: Levallorphan is known to affect adsorption of the male specific phage MS-2 to the pili. The

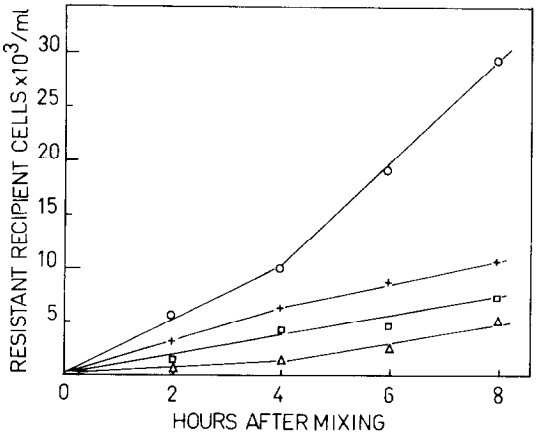

Fig. 1 Inhibition of R-factor transfer by levallorphan. Donor and recipient cells from stationary phase cultures were mixed in presence of o—o no levallorphan, +—+ 0.6 mM, □—□ 0.8 mM, Δ—Δ 1.0 mM levallorphan. At time intervals aliquots of the culture mixture were plated on antibiotics agar (kanamycin, neomycin, chloramphenicol and streptomycin) and the resistant recipient cells were counted.

conjugation necessary to allow transfer of R-factors also depends on the existence, and probably on a correct functioning of the pili. In fig. 1 it is shown that levallorphan is also able to inhibit the manifestation of R-factors in recipient cells. This inhibition is comparable to the inhibition of phage infection by MS-2 phages insofar as it can be observed at a concentration of 0.7 mM of levallorphan, not sufficient to inhibit the growth of the bacteria significantly in Penassay broth. The inhibition is concentration dependent (Fig. 1).

b) Spontaneous loss of R-factors: The inhibition of the transfer of the R-factor by levallorphan could be due to increased loss of R-factors either in the donor cells or in the recipient cells. Therefore a penicillin screening technique similar to that for isolation of auxotropic mutants was employed. With this technique only cells which have become sensitive to the corresponding antibiotics should be isolated, i.e. such cells which have lost the R-factor responsible for the resistance (Tab. 2).

TABLE 2

	Addition of 0.7 mM Levallorphan	Percent of Cells which Survived Penicillin Treatment in Presence of		
		Kanamycin	Kanamycin Neomycin	Kanamycin Neomycin Chloramphenicol
Proteus Rettgeri R^+	-	1.5×10^{-1}	3.0×10^{-2}	1.4×10^{-3}
	+	1.8×10^{-1}	1.3×10^{-2}	3.0×10^{-3}
E.coli W 677, R^+	-	4.1×10^{-2}	4.6×10^{-2}	7.5×10^{-4}
	+	2.3×10^{-2}	6.3×10^{-2}	9.3×10^{-4}

The number of surviving cells was higher when only kanamycin or kanamycin and neomycin were used to avoid outgrowth of sensitive cells. However when we checked these cells for their resistance, most of them were resistant to the corresponding antibiotic on antibiotic agar. Probably this is due to so called persisters (Lamanna and Malette (7)) arising during penicillin treatment. This phenomenon was not observed if all three antibiotics were employed together and the cells were tested for their resistance. Therefore the survivors in the case in which kanamycin, neomycin and chloramphenicol were used together as selective agents show a more realistic result. Levallorphan in this concentration does neither stimulate the spontaneous loss of the R-factor markedly nor does it inhibit, the differences being partially due to statistical error. Therefore the reason for the inhibition was presumed to be at another step of the transfer.

c) Kinetics of transfer for the different resistance markers: It has been reported by Watanabe (8) that the kinetics of transfer of different resistant markers is so that all markers are transferred en bloc. Anderson and Lewis (9) described a transfer system in which the different markers were transferred at different time-intervals. They suggest that these markers may reside on different episomes. Fig. 2 shows that in our system the transfer of the different markers also appeared after different time-intervals. We have not yet been able to distinguish clearly whether this is due to different gene loci on one episome or to different episomes. The transfer in our system is much slower than that usually described for E.coli-systems (10), so that it might be possible to observe time dependent transfer in our system. Further investigations along this line are planned.

In any case however, as can be seen in Fig. 2 the initial rates of transfer for the markers of kanamycin and neomycin resistance are not significantly different in the presence of levallorphan compared with the controls. The chloramphenicol determinant is an exception as it shows a lag in the presence of levallorphan. But the rate of transfer of the chloramphenicol markeris only slightly smaller compared to the control.

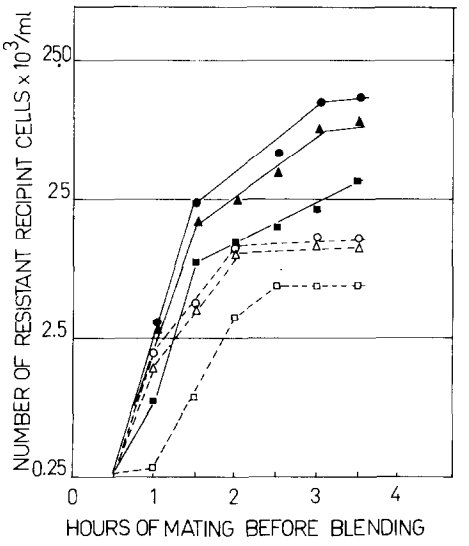

Fig. 2 Appearance of the different resistance markers after mixing of donor and recipient cells in presence and absence of levallorphan. At the time intervals indicated aliquot samples were taken and plated on agar containing 70 µg/ml of kanamycin ●—●, 70 µg/ml of kanamycin and 100 µg/ml of neomycin ▲—▲, and 70 µg/ml of kanamycin, 100 µg/ml neomycin and 20 µg/ml chloramphenicol ■—■. In addition all agar plates contained 500 µg/ml streptomycin. The open symbols and dashed lines represent the surviving recipient cells on the same antibiotic agars just mentioned from a culture mixture containing 0.7 mM levallorphan.

The control mixture without levallorphan does not attain a plateau in the time concerned while that of the mixture treated with levallorphan does. We assume that in the latter case there are less pairs formed which can transfer their episome(s).

d) Pair formation in presence of levallorphan: These results prompted us to investigate the formation of mating-pairs in the presence of levallorphan. The technique we used is based on the idea that formed mating-pairs can be counted at the time of sampling by diluting the mixture to such an extent that further pair formation becomes improbable (11). Two conditions have to be fulfilled for such an experiment. First the mating-pairs must be able to withstand pipetting, centrifugation and gentle shaking and secondly, each pair should lead to a resistant recipient colony, i.e. the transfer and manifestation of the episome must be expressed in the recipient. From the previous findings concerning the kinetics of the transfer one can conclude that the transfer of the episome itself is not significantly inhibited. The resistance of the pairs to pipetting, centrifugation and gentle shaking was also checked and found not to be affected. Fig. 3 therefore leads to the following conclusion: since the resistant recipient colonies decrease after addition of levallorphan and since the transfer of

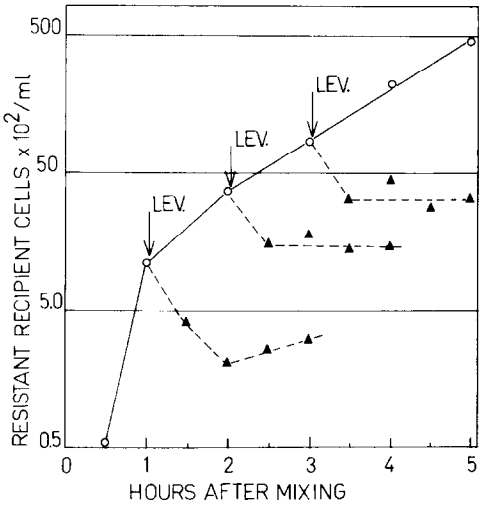

Fig. 3 Formation of mating-pairs in presence of levallorphan.
A donor and recipient cell mixture was divided at the time intervals indicated by the arrows. The control samples O—O were directly plated onto antibiotics agar (kanamycin, neomycin, chloramphenicol, streptomycin). The other part of the mixture received levallorphan in a concentration of 0.7 mM. After the indicated time intervals samples of this mixtures were diluted 1:500 in fresh Penassay broth, allowed to stand for 60 minutes at room temperature, centrifuged at 15 000 RPM and then plated on the above mentioned antibiotics agar. ▲—▲ resistant recipient cells representing the number of mating-pairs at time of sampling.

the episome is hardly affected, mating-pairs which had already formed, must have come apart in the presence of levallorphan.

DISCUSSION - Levallorphan, a non narcotic derivative of morphine, has been shown to have a series of effects on bacteria (12, 13, 14, 18, 19), phages (15, 3) and viruses (16). We have recently found that levallorphan affects adsorption of MS-2 phage to the sex-pili and that even a desorption of already adsorbed phage occurs in the presence of levallorphan (3). We were interested to establish whether these findings are specific for the RNA phage or whether the effect is due to an action concerning the pili in general. From data of Simon et al. (14), Greene and Magasanik (17), Boquet et al. (18) and from own data (unpublished) a change in the permeability of the cell membrane can be deduced. However, the experiments cited were done, in part, with high concentrations of the drug. We worked at concentrations hardly affecting the growth of the bacteria. At this concentration the effects on permeability seem to be different as far as the loss of ATP (14 and own observations) and of thiomethylgalactoside is concerned. However, under these conditions the drug shows a significant inhibitory action on the transfer of R-factors. From our results we have to conclude that mainly the formation of mating-pairs is affected. This conclusion gains further support from the fact that the inhibition of transfer of R-factors can be reversed by Mg^{2+} (in preparation). This ion plays an essential role in the adsorption of RNA phage. It was also shown by Boquet et al. (18) that it protects resting cells from being killed even at high levallorphan concentrations. We therefore conclude that levallorphan brings about damage to the pili and probably to the whole cell membrane. This damage, in turn, is also responsible for the inhibition of formation of mating-pairs but does not affect the energy-dependent transfer of the episome.

ACKNOWLEDGEMENTS - We wish to thank Dr. A. Bauernfeind for diagnosting the Proteus Rettgeri strain, Dr. D. Jarvis for reviewing the manuscript and Prof. K. Liebermeister for providing a grant to one of us (R.L.).

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