Arch. Microbiol. 100, 105—114 (1974)

Edeine Inhibition and Resistance in *Neurospora*

Margit Wagenmann and Walter Klingmüller

Institut für Genetik der Universität München

Walter Neupert

Institut für Physiologische Chemie und Physikalische Biochemie der Universität München

Received August 1, 1974

Abstract. To obtain data on the biochemical effects of edeine in the fungus *Neurospora crassa*, *in vivo* protein synthesis, *in vitro* protein synthesis, as well as *in vivo* RNA and DNA synthesis of the wildtype and an edeine resistant mutant were measured.—Incorporation of ^{3}H leucine into conidia of both strains, which served as a measure for *in vivo* protein synthesis, was inhibited by 200 µg edeine/ml as follows: Wildtype approx. 40%, mutant approx. 6%.—Incorporation of ^{14}C phenylalanine into polyphenylalanine in a cell free system with ribosomes from either the wildtype or the mutant, was inhibited between 74 and 95% by edeine at a ratio of 2 molecules edeine per ribosome.—Incorporation of ^{3}H adenosine into conidia, serving as a measure for *in vivo* RNA synthesis, was inhibited in the wildtype (approx. 30% inhibition by 200 µg edeine/ml). It was, however, not influenced in the *edr* mutant. Similarly, *in vivo* DNA synthesis was decreased in the wildtype, but not in the mutant.—These results suggest that edeine acts at more than one site. The resistance of the mutant *edr*-29 (*edr*-2 locus) is tentatively interpreted as due to a block in edeine uptake.

Key words: Edeine — *Neurospora* — Edeine-Resistance — Protein-Synthesis — DNA-Synthesis — RNA-Synthesis — Fungi.

Edeine is an oligopeptide antibiotic from *Bacillus brevis*. It is a mixture of two components, edeine A and B, each of which is composed of 5 amino acids and 1 molecule of spermidine, edeine B having a guanyl group linked to the spermidine residue (Hettinger and Craig, 1970). Edeine has a broad action spectrum, it inhibits growth of yeasts and fungi, but also of gram-negative and -positive bacteria (Kurylo-Borowska, 1967). *In vivo* experiments have shown that in *Escherichia coli* there is a strong inhibition of DNA synthesis, whereas RNA and protein synthesis are barely affected (Kurylo-Borowska, 1962, 1964). In cell free systems, with ribosomes from *E. coli* and from rabbit reticulocytes, a strong inhihition of protein synthesis was observed (Hierowsky and Kurylo-Borowska, 1965; Obrig *et al.*, 1971).

It has been shown that edeine in *E. coli* influences genetic recombination (Piekarowicz *et al.*, 1969). In addition, recombination frequencies in this bacterium are altered in edeine resistant strains (Wlodarczyk

et al., 1969). Corresponding data were recently reported for *Neurospora*, where edeine resistant mutants have been obtained and used for such investigations (Beetz and Klingmüller, 1973; Teles-Grilo and Klingmüller, 1974). Hence, a detailed knowledge of the biochemical effects of edeine in *Neurospora* would be useful not only for studies on the mechanism of DNA synthesis, but also for studies on the mechanism of genetic recombination in eukaryotes. This lead us to investigate the effects of edeine on *in vivo* and *in vitro* protein synthesis, on *in vivo* RNA, and on *in vivo* DNA synthesis of the wildtype and an edeine resistant mutant of *Neurospora crassa*.

Material and Methods

1. Strains. Neurospora crassa wildtype 74-OR 23-1A de Serres; origin: Dr. Mary E. Case, Yale University. *Neurospora crassa* edeine resistant mutant *ed*^{r}-29/18 (*ed*^{r}-2 locus); origin: M. Leonor Teles-Grilo, this institute (see Teles-Grilo and Klingmüller, 1974, for details).

2. Special Chemicals. Edeine complex, Pharmaceutical Works "Polfa", Tarchomin, Poland. In the present experiments this drug was applied at 200 µg/ml (*in vivo*) resp. 2 µg/ml (*in vitro*). L-leucine ^{3}H, spec. act. 1 Ci/mMol; L-phenylalanine ^{14}C, spec. act. 513 mCi/mMol; adenosine ^{3}H, spec. act. 12.1 Ci/mMol; The Radiochemical Centre, Amersham, England. Components of incubation mixture for *in vitro* protein synthesis: Poly-U and pyruvate kinase, Boehringer, Mannheim; *t*RNA^{Phe} from yeast, Dr. R. Thiebe, Institut für Physiologische Chemie der Universität München; phenylalanyl-*t*RNA-synthetase from yeast, Dr. R. Hirsch, same institute; AMT buffer: 0.1 M NH_4Cl, 0.01 M $MgCl_2$ and 0.01 M Tris-HCl, pH 7,6.

3. Growth Media. a) Solid: Glycerol complete after Horowitz (1947); Difco Bacto Neurospora Culture Agar. b) Liquid: Fries' minimal after Beadle and Tatum (1941); Vogels minimal after Vogel (1956); sucrose medium: 20 ml Vogels (50 times) and 20 g sucrose in 1 l H_2O; peptone medium: 5 g Difco Peptone, 5 g Difco Yeast Extract, 15 g malt extract, Merck and 20 ml Vogels minimal (50 times) in 1 l H_2O.

4. Procedures for Obtaining Conidia and Conidial Suspensions. a) Wildtype conidia and conidial suspensions were obtained as described by Neuhäuser *et al.* (1970). The basic medium was glycerol complete. b) If growth media without edeine were used to obtain conidia of the *ed*^{r} mutant, only part of the conidia that came up had the resistant phenotype (Teles-Grilo and Klingmüller, 1974). To enforce expression of the resistant phenotype in all conidia, the mutant had to be grown in the presence of edeine. Edeine, however, is not tolerated in combination with glycerol complete, hence the basal medium for these experiments was *Neurospora* Culture Agar. Edeine was added to give a concentration of 200 µg/ml.

5. Protein Synthesis in vivo. 2.4×10^7 conidia each were filtered off onto millipore filter disks, washed twice with H_2O, and resuspended in flasks with 20 ml Fries' minimal, with or without 200 µg edeine/ml. Here they were aerated for 10 min at 25°C ("edeine pretreatment"). Then, 0.5 µCi ^{3}H leucine were added per ml. After incubation periods up to 60 min, samples of 2 ml were transferred into test tubes, containing 0.5 ml 50% TCA and 0.5 ml 0.1 M unlabeled leucine, to stop the incoporation of the radioactive leucine. The mixtures were kept at 90°C for 30 min. Then the cells were filtered off and washed twice with H_2O. They were dried on the filters and the radioactivity was measured.

Kurylo-Borowska (1962) had found in *Escherichia coli* that *in vivo* protein synthesis is not affected before a 3 hrs pretreatment of the cells with edeine. We

have checked this point in *Neurospora* in experiments, where the original pretreatment of 10 min was extended by up to 3 hrs. The results of such experiments (Fig. 1; Wagenmann, 1974) show that the inhibition of leucine incorporation in the presence of edeine indeed increases with increasing duration of edeine pretreatment. However, this increase is relatively small. Since at several hours of pretreatment of the conidia harmful side effects were to be expected, and the inhibition at 10 min pretreatment proved strongly significant, this short edeine pretreatment was chosen in all later experiments.

6. Protein Synthesis in vitro. Ribosomes were prepared and poly-U dependent polyphenylalanine synthesis was measured as described before (Pongratz and Klingmüller, 1973). The edeine resistant phenotype is unstable in all ed^r-mutants checked so far (as opposed to the genotype). It is maximally expressed in cells grown in the presence of the drug (Teles-Grilo and Klingmüller, 1974). For this reason, ribosomes from the ed^r mutant used here had to be prepared from mycelia grown in the presence of edeine. In liquid sucrose medium, which was used to obtain mycelia and ribosomes from the wildtype, the ed^r mutant does not tolerate edeine to the extent it does on solid medium. However, this mutant tolerates 130 µg edeine/ml in liquid peptone medium. Hence, for growth of ed^r mycelia this medium was chosen. As a control, in parallel runs, the wildtype was not only grown in liquid sucrose medium, but also in peptone medium either without or with a limiting concentration of edeine. Hence, ribosomes from four different types of mycelia were prepared and used in the *in vitro* tests, viz.:

mycelia of type 1: wildtype grown in sucrose medium,
mycelia of type 2: wildtype grown in peptone medium,
mycelia of type 3: wildtype grown in peptone medium plus 75 µg edeine/ml, and
mycelia of type 4: ed^r mutant grown in peptone medium plus 130 µg edeine/ml.

7. RNA and DNA Synthesis in vivo. Since fungi incorporate exogeneous thymidine very inefficiently (Jannsen *et al.*, 1970), in the experiments to be described here ^{3}H adenosine was used. This substance, if added to whole cells, is incorporated into both RNA and DNA (Hartwell, 1967; Groß, in preparation). Pilot runs had shown that incorporation was optimal if conidia (1.2×10^8/ml) were germinated in Vogels minimal and 2% glucose for 4 hrs at 25° C under constant aeration. Hence, such conidia were used. They were, in parallel runs, either supplied with edeine (200 µg/ml) for 10 min, or left without it. Then, 16 µCi ^{3}H adenosine were added per ml. Aliquots of 2.5 ml were taken at various time intervals up to 60 min, mixed with unlabeled adenosinesulfate (final conc. 0.8 M) and TCA (final conc. 7%), and kept at 0° C for 1 hr. They were then filtered off, washed twice with H_2O, dried and counted. According to Hartwell (1967) and Groß (in preparation), ^{3}H adenosine predominantly labels RNA; labelling of DNA figures to maximally 10% of total label. To find the amount of radioactivity in DNA, samples withdrawn from the incubation mixtures were treated with 2.5 ml 1 N NaOH for 16 hrs, to hydrolyze RNA, before adding unlabeled adenosinesulfate and TCA.

8. Statistics. Deviations indicated in Tables 1 and 2 and Figs. 1, 7 and 9 are standard deviations from the mean. Where based on percent values, angular transformation was used (Fisher and Yates, 1953).

Results

Influence of Edeine on *in vivo* Protein Synthesis

Incorporation of ^{3}H leucine into proteins of conidia of the wildtype and of ed^r mutant Nr. 29/18 (ed^r-2 locus) was measured in the presence and

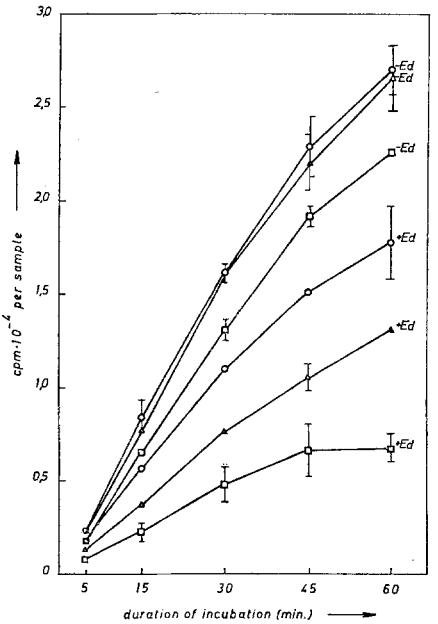

Fig. 1. Influence of edeine on *in vivo* protein synthesis of wildtype conidia pretreated with edeine for various periods of time. Pretreatment periods: o = 10 min, ▵ = 70 min, □ = 190 min. —Ed and +Ed: Pretreatment and incubation without or with edeine. At the end of the pretreatment period, ^{3}H leucine was added to the incubation mixture, samples were taken at the intervals indicated, and processed as described in "Material and Methods". The radioactivity of the samples is plotted against incubation time in the presence of ^{3}H leucine

Table 1. Inhibition of protein synthesis by edeine in wildtype and mutant conidia. 10 min pretreatment, 200 μg edeine/ml; protein synthesis was measured as incorporation of ^{3}H leucine into acid precipitable material. Data are averages from 3 experiments each

Duration of incubation with ^{3}H leucine (min)	% Inhibition	
	wildtype	mutant
5	41 ± 0.6	6.3 ± 2.7
20	48 ± 0.8	6.0 ± 2.8
45	52 ± 0.8	16.6 ± 2.2

absence of edeine. The results are shown in Table 1. Protein synthesis of the wildtype was inhibited by the drug between 41% (5 min) and 52% (45 min of incubation). In contrast, that of the mutant was inhibited to a much lower degree (6—17%).

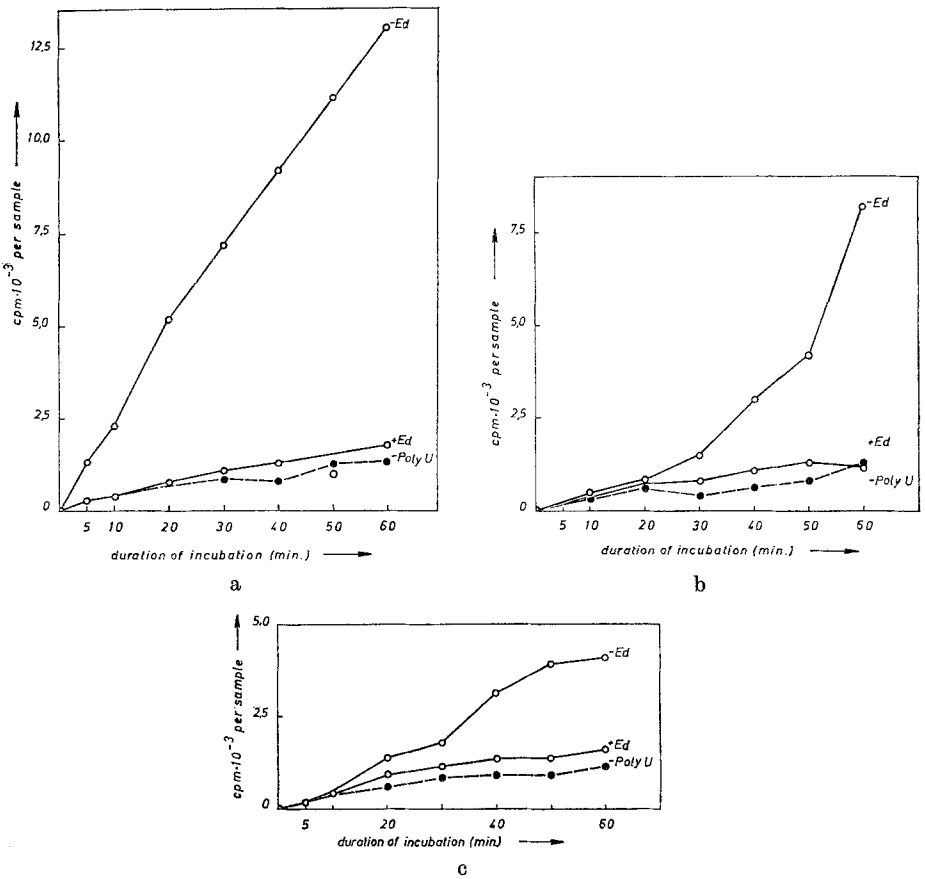

Fig. 2a—c. Influence of edeine on *in vitro* protein synthesis of the wildtype. (a) Ribosomes from mycelial type 1 (sucrose medium). (b) Ribosomes from mycelial type 2 (peptone medium). (c) Ribosomes from mycelial type 3 (peptone medium plus 75 μg edeine/ml). — *Poly U:* Incubation without poly U and edeine

Influence of Edeine on *in vitro* Protein Synthesis

To study the effect of edeine on ribosomes, the synthesis of polyphenylalanine in a poly-U directed reconstituted system of protein synthesis was measured. Ribosomes from 3 different wildtype mycelia (mycelia of type 1—3) and ribosomes from *ed*^{r} mycelia (mycelia of type 4) were used. Figs. 2 and 3 give the results of such experiments. In experiments without edeine in the incubation assay, incorporation of radioactive phenylalanine into polyphenylalanine was higher with ribosomes from mycelia of types 1 and 2 (grown without edeine) than with ribosomes from mycelia of types 3 and 4 (grown in the presence of edeine). This

means that growth of mycelia in the presence of edeine leads to alterations or damage of their ribosomes. If edeine is present in the incubation assay, a strong inhibition is observed with ribosomes from wildtype as well as from edeine resistant cells. The pooled data from several experiments are given in Table 2. Percent inhibition (H) was calculated as follows:

$$H\ (\%) = \frac{(a - b) \times 100}{a}$$

Table 2. Inhibition of *in vitro* protein synthesis by edeine (2 μg/ml) for ribosomes from 4 different mycelial types. Data are averages from 2—4 experiments each

Ribosomes from	Inhibition H (%)
Mycelial type 1 (wildtype, sucrose medium)	95 ± 0.3
Mycelial type 2 (wildtype, peptone medium)	88 ± 5.5
Mycelial type 3 (wildtype, peptone medium plus 75 μg edeine/ml)	75 ± 4.0
Mycelial type 4 (*ed*^{r} mutant, peptone medium plus 130 μg edeine/ml)	84 ± 3.3

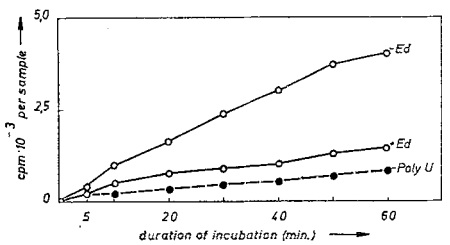

Fig. 3

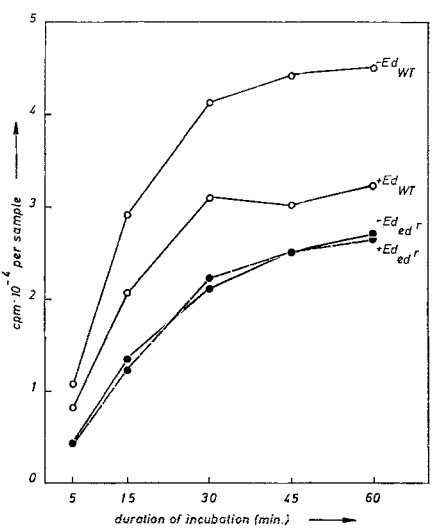

Fig. 4

Fig. 3. Influence of edeine on *in vitro* protein synthesis of the *ed*^{r}-29 mutant. Ribosomes were taken from mycelial type 4 (peptone medium plus 130 μg edeine/ml). Abbreviations as in Fig. 2

Fig. 4. Influence of edeine on *in vivo* RNA synthesis of wildtype and mutant conidia. Pregerminated cells were incubated with ^{3}H adenosine. Radioactivity of samples is plotted against time of incubation. + *Ed* = 200 μg edeine/ml. *Wt* wildtype; *ed*^{r} mutant

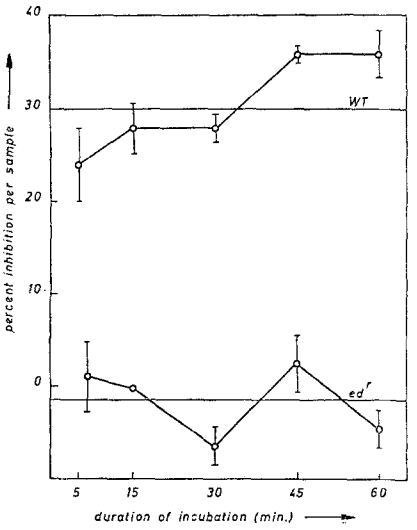

Fig. 5

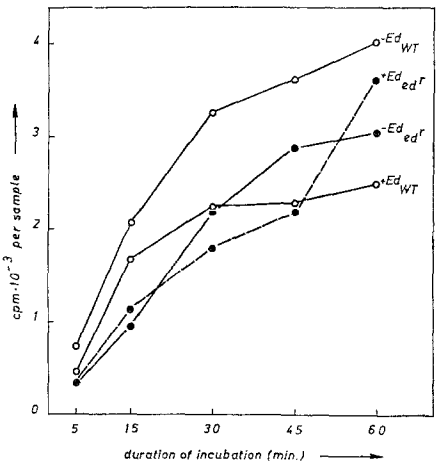

Fig. 6

Fig. 5. Inhibition of *in vivo* RNA synthesis in the wildtype and the mutant by edeine. Average inhibition is plotted against duration of incubation with ^{3}H adenosine. Averages were derived from 3—5 experiments each. The horizontal lines indicate the two means, which were obtained by pooling the data from the different times of incubation

Fig. 6. Influence of edeine on *in vivo* DNA synthesis of wildtype and mutant conidia. Conditions and abbreviations as in Fig. 4

where a = increase in radioactivity without edeine between 20 and 50 min incubation, corrected for increase in radioactivity without poly-U, and b = increase in radioactivity with edeine in the same interval, corrected for increase in radioactivity without poly-U. The data substantiate that the ribosomes from wildtype and mutant are inhibited to the same extent. It is concluded that mutation *ed*^{r}-29 does not cause resistance via an alteration of ribosomes.

Influence of Edeine on RNA and DNA Synthesis

Incorporation of ^{3}H adenosine into conidia of the wildtype and of the *ed*^{r} mutant was measured with and without edeine. Fig. 4 shows the incorporation of this precursor into RNA and Fig. 6 into DNA. It can be seen from Fig. 4 that RNA synthesis of the wildtype is inhibited by edeine. In contrast, RNA synthesis of the mutant is not. The same holds for DNA synthesis (Fig. 6). Since incorporation of the label varied considerably from experiment to experiment, for a precise evaluation of our results, data from several repeats were pooled. In Figs. 5 and 7 the per-

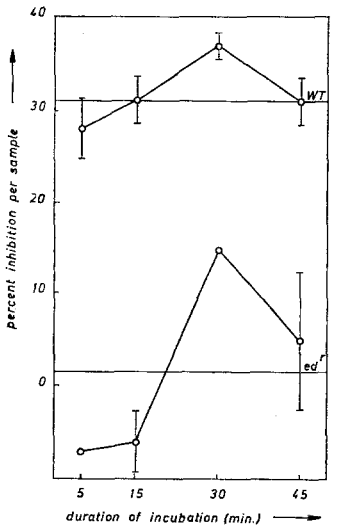

Fig. 7. Inhibition of *in vivo* DNA synthesis in the wildtype and the mutant by edeine. Conditions and abbreviations as in Fig. 5

cent of inhibition of RNA and DNA synthesis by edeine in wildtype and mutant is plotted against duration of incubation. It can be seen that RNA synthesis is inhibited by edeine in the wildtype (approx. 30% inhibition with 200 µg edeine/ml), but not in the mutant. Quite similar values are observed for DNA synthesis.

Discussion

The experiments described here have shown that incorporation of leucine into proteins of conidia of *Neurospora* is inhibited by edeine in the wildtype, but not in the edeine resistant mutant *ed*^{r}-29. On the other hand, the sensitivity of isolated ribosomes to this drug is the same with the wildtype and with the mutant. Since wildtype and mutant ribosomes are equally affected *in vitro*, it is concluded that edeine resistance of protein synthesis in mutant *ed*^{r}-29 is not caused by an alteration of the ribosomes. Obviously, this resistance is based on a different mechanism than that in the cycloheximide resistant mutants described previously (Pongratz and Klingmüller, 1973). In these latter mutants, the ribosomes have lost the sensitivity to the antibiotic.

With regard to DNA and RNA synthesis in cells, it is shown here that in the wildtype both reactions are inhibited by edeine, whereas in the mutant they are not affected. Hence, it appears that in all experi-

ments on DNA, RNA and protein synthesis with intact cells, edeine has an inhibitory effect in the wildtype but not in the mutant. Therefore, the most likely cause of edeine resistance in the mutant is an alteration of edeine uptake, such that cells of the mutant take up less edeine than those of the wildtype.

Such an alteration could for instance result from a defect in a transport system for oligopeptides. Another possibility would be that in the mutant an edeine degrading enzyme (peptidase) is effective. However, the latter possibility is ruled out by the finding that mutation *edr*-29 is recessive to the wildtype allele in heterokaryon tests (Teles-Grilo and Klingmüller, 1974; Klingmüller, 1967). In contrast, the first explanation is supported by this finding.

Acknowledgements. The authors thank Mr. A. Pfaller for valuable help in carrying out experiments with the reconstituted system.

Note Added in Proof. In the above experiments, the influence of edeine on protein synthesis was measured *in vivo* and *in vitro*, but on RNA and DNA synthesis could only be checked *in vivo*. For setting up an in vitro system on RNA synthesis, RNA polymerases of *Neurospora* would be needed. These are not available yet. However, RNA polymerases of yeast, a related organism, have recently been isolated and purified by several groups of workers. W. Klingmüller could therefore check the influence of edeine on RNA synthesis *in vitro*, using RNA polymerases of *Saccharomyces carlsbergensis*, in the Biochemisch Laboratorium, Vrije Universiteit of Amsterdam (Director: Prof. Dr. R. J. Planta), during the tenure of an EMBO short term fellowship, in cooperation with Drs. H. van Keulen and Dr. J. Retèl. Transcription of phage λ DNA, phage φ 80 DNA, and calf thymus DNA were measured with the A- and B-enzyme, without edeine, or in the presence of 10, resp. 100 µg edeine/ml, using incorporation of ^{3}H. UTP into TCA precipitable material as criterium. In these experiments it was found that RNA synthesis in vitro is not affected by the drug.

References

Beadle, G. W., Tatum, E. L.: Genetic control of biochemical reactions in *Neurospora*. Proc. nat. Acad. Sci. (Wash.) **27**, 499–506 (1941)

Beetz, B., Klingmüller, W.: High recombination rates in the presence of edeine. Naturwissenschaften **60**, 301 (1973)

Fisher, R. S., Yates, F.: Statistical tables for biological, agricultural and medical research, 4th ed., p. 55. New York: Hafner 1953

Hartwell, L. H.: Macromolecule synthesis in temperature sensitive mutants of yeast. J. Bact. **93**, 1662–1663 (1967)

Hettinger, T. P., Craig, L.: Edeine. IV. Structures of the antibiotic peptides, edeine A_1 and B_1. Biochemistry **9**, 1224–1232 (1970)

Hierowsky, M., Kurylo-Borowska, Z.: On the mode of action of edeine. Effect of edeine on the synthesis of polyphenylalanine in a cell free system. Biochim. biophys. Acta (Amst.) **95**, 578–589 (1965)

Horowitz, N. H.: Methionine synthesis in *Neurospora*. The isolation of cystathionine J. biol. Chem. **171**, 255–264 (1947)

Jannsen, S., Lochmann, E. R., Megnet, R.: Specific incorporation of exogenous thymidine monophosphate into DNA in *Saccharomyces cerevisiae*. FEBS Letters **8**, 113–115 (1970)

Klingmüller, W.: Analyse der Sorboseresistenz von *Neurospora crassa* an Heterokaryen sorboseresistenter Mutanten; ein Beitrag zur Genetik des aktiven Transports, I. Molec. Gen. Genetics **100**, 117—139 (1967)

Kurylo-Borowska, Z.: On the mode of action of edeine. Biochim. biophys. Acta (Amst.) **61**, 897—902 (1962)

Kurylo-Borowska, Z.: On the mode of action of edeine. Effect of edeine on the bacterial DNA. Biochim. biophys. Acta (Amst.) **87**, 305—313 (1964)

Kurylo-Borowska, Z.: Edeine. In: Antibiotics, D. Gottlieb and P. D. Shaw, eds., Vol. 2, pp. 342—352. Berlin-Heidelberg-New York: Springer 1967

Neuhäuser, A., Klingmüller, W., Kaudewitz, F.: Selektion Actidion-resistenter Mutanten bei *Neurospora crassa* sowie ihre genetische und biochemische Analyse. Molec. Gen. Genetics **106**, 180—194 (1970)

Obrig, T., Irvin, J., Culp, W., Hardesty, W.: Inhibition of peptide initiation on reticulocyte ribosomes by edeine. Europ. J. Biochem. **21**, 31—41 (1971)

Piekarowicz, A., Groniowska, E., Kunicki-Goldfinger, W.: Mechanism of conjugation and recombination in bacteria. V. Effect of edeine on F^+ cells. Acta microbiol. pol. **1A**, 15—22 (1969)

Pongratz, M., Klingmüller, W.: Role of ribosomes in cycloheximide resistance of *Neurospora* mutants. Molec. Gen. Genetics **124**, 359—363 (1973)

Teles-Grilo, M. L., Klingmüller, W.: Induction and genetical analysis of edeine resistant mutants of *Neurospora crassa*. Molec. Gen. Genetics **133**, 123—133 (1974)

Vogel, H. J.: A convenient growth medium for *Neurospora*. Microbial gen. Bull. **13**, 42—43 (1956)

Wagenmann, M.: Wirkung von Edein auf die *in vivo* und in *vitro* Proteinsynthese, sowie die *in vivo* RNS und DNS Synthese des Wildtypes und einer Edein-resistenten Mutante von *Neurospora crassa*. Diplomarbeit, Universität München 1974

Wlodarczyk, M., Najer, A., Piekarowicz, A., Kunicki-Goldfinger, W.: The mechanism of conjugation and recombination in bacteria. VII. Further characteristic of recombination deficient *Hfr* mutants. Acta microbiol. pol. **1A**, 123—124 (1969)

Prof. Dr. Walter Klingmüller
Institut für Genetik
der Universität
D-8000 München 19
Maria Ward-Str. 1a
Federal Republic of Germany