INHIBITION BY LOMOFUNGIN OF NUCLEIC ACID AND PROTEIN SYNTHESIS IN *SACCHAROMYCES CEREVISIAE*

M. CANNON*, J.E. DAVIES and A. JIMENEZ**
Department of Biochemistry, University of Wisconsin, Madison, Wisc. 53706, USA

Received 20 March 1973
Revised version received 30 March 1973

1. Introduction

The phenazine antibiotic lomofungin [1] inhibits the growth of many organisms [2] and it has been suggested that its primary mode of action is on the synthesis of RNA [2]. In the present investigation we have studied the effect of the drug on nucleic acid and protein synthesis in spheroplasts of *Saccharomyces cerevisiae*. Spheroplasts can carry out rapid synthesis of RNA and protein [3] and since they can be lysed in detergent easily and quickly this has allowed us to study, by sucrose gradient analysis, polyribosome profiles before and after treatment of spheroplasts with the drug.

Our results show that lomofungin, at a concentration of 5 μg/ml, rapidly inhibits the incorporation of uridine into RNA. This effect is not reversed by extensive washing of either spheroplasts or whole cells. The drug also inhibits the synthesis of DNA in whole cells. In contrast, protein synthesis in spheroplasts is at first unaffected by lomofungin which only becomes inhibitory approx. 20 min after its addition. Analysis of polyribosome profiles for various time points after addition of lomofungin indicates that the drug induces complete breakdown of polyribosomes over a 45 min incubation. The gradual disappearance of the polyribosome profile corresponds in time approximately with the inhibition of amino acid incorporation into protein. The decay of protein synthetic capacity which follows inhibition of nucleic acid synthesis occurs with a half life of approx. 20–25 min.

2. Materials and methods

S. cerevisiae strain Y166 (trp5 his4 MA1) was grown in synthetic complete medium [4] at 30° with shaking. Solid media for strains and cell-viability determinations contained 1% yeast extract, 2% Bactopeptone, 2% glucose and 2% agar. Cell growth was followed at an absorbance of 550 nm in a Bausch and Lomb Spectronic 20. A reading of 0.30 approximated 10^7 cells/ml. Viability of cell cultures was determined by serial dilution and plating on solid media; plates were scored after 48 hr incubation at 30°.

Spheroplasts were prepared as described previously [3, 5].

RNA and protein synthesis were monitored using, respectively, ^{3}H-labelled leucine or ^{14}C-labelled methionine. At various times, samples were added to ice-cold trichloroacetic acid (TCA). To measure amino acid incorporation into protein, samples were held at 90–95° for 15 min. All precipitates were collected on glass fibre circles (Whatman GF/C 2.4 cm), washed 7 times with ice-cold 5% TCA, dried and counted in a toluene based scintillation fluid.

To analyze polyribosome profiles, spheroplasts were inhibited by cycloheximide, poured onto frozen M sorbitol and spheroplasts collected by centrifugation at 8,000 *g* for 5 min in a Sorvall SS-34 rotor. The pellets were resuspended in Tris-HCl buffer, pH 7.4

* On leave of absence from Department of Biochemistry, University of London King's College, Strand, London W.C. 2, England.
** Present address: C.S.I.C. Centro de Investigaciones Biologicas, Instituto de Biologia Celular, Velazquez, 144, Madrid-6, Spain.

(0.25 ml), containing 30 mM magnesium acetate and 80 mM KCl, and 5% sodium deoxycholate (25 μl) and 5% Brij 58 (40 μl) were added to lyse spheroplasts. Lysates were layered on top of 5 ml 10–30% sucrose gradients in the above buffer and centrifuged for 35 min at 35,000 rpm in a Spinco model SW50.1 rotor. Gradients were analyzed at 254 nm in an Isco model DUA-2 gradient analyzer.

[5,6-^{3}H]Uridine (42.4 Ci/mM) and [4,5-^{3}H]L-leucine (64 Ci/mM) were obtained from the New England Nuclear Corporation, Boston, Massachusetts. [Methyl-^{14}C]L-methionine, 60 mCi/mM, was obtained from The Radiochemical Centre, Amersham. Lomofungin and cycloheximide were both kindly supplied by Dr. G.B. Whitfield, Jr. of the Upjohn Company, Michigan. Lomofungin was dissolved freshly in dimethyl sulfoxide (DMSO) for each experiment. Glusulase was obtained from Endo Laboratories, Inc., Garden City, New York.

3. Results and discussion

The effect of lomofungin at low concentration (5 μg/ml) on the viability of *S. cerevisiae* has been studied. The drug was added to a yeast culture growing exponentially (approx. 10^7 cells/ml) and incubation was continued at 30°. Samples were then removed at 0.5 hr, 1 hr, and 5 hr, serially diluted with distilled water and aliquots cultured to determine the number of growing colonies. Treatment with lomofungin for only 0.5 hr resulted in a 95% reduction in the number of viable cells. This figure rose to 100% with cells exposed to 20 μg/ml of lomofungin for 0.5 hr.

To determine the effect of lomofungin on RNA synthesis in *S. cerevisiae*, spheroplasts were incubated with ^{3}H-labelled uridine and samples removed at various times into 10% TCA. Lomofungin (5 μg/ml) was added 15 min after the start of the incubation. This allows time for added ^{3}H-labelled uridine to equilibrate with the intracellular pool of nucleic acid precursors and ensures linear incorporation of uridine at the time of drug addition. The results are shown in fig. 1. Uptake of uridine into RNA is inhibited within 3 min of addition of lomofungin.

RNA synthesis in intact yeast cells was assayed to determine if the inhibitory effect of lomofungin is

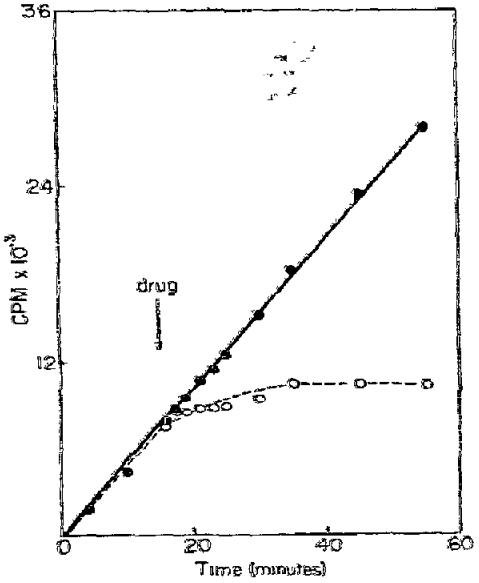

Fig. 1. Uptake of uridine by yeast spheroplasts and the effect of lomofungin. A culture (30 ml) of *S. cerevisiae* was harvested at an absorbance of 0.32 measured at 550 nm. The cells were washed in water (30 ml) and resuspended in M sorbitol (3 ml). Glusulase (30 μl) was added and the suspension incubated at 30° with gentle shaking. After 45 min the suspension was diluted into synthetic complete medium (60 ml) and shaken gently at 30° for 2.5 hr. Samples (10 ml) were mixed with ^{3}H-labelled uridine (25 μCi) and cold uridine (10 μg) and samples (0.5 ml) removed into ice-cold 10% TCA (0.5 ml) containing 0.25 mg/ml uridine, at the times indicated. Fifteen min after starting the incubation DMSO (10 μl) was added to one sample and a solution of lomofungin in DMSO (10 μl of 5 mg/ml) added to the second sample. Further aliquots were removed as indicated. Samples were processed and counted as described in Materials and methods. (●—●—●) = Control; (o--o--o) = sample with lomofungin added at the time indicated.

reversible. One sample of a growing culture was stored on ice and a further sample incubated for 0.5 hr with lomofungin (5 μg/ml). Both samples were harvested, washed several times, resuspended in medium and incubated for 20 min at 30°. ^{3}H-labelled uridine was then added and samples of the cultures removed into 10% TCA at various times. The control culture incorporated uridine into RNA rapidly after a short lag phase. Cells previously treated with lomofungin remained inhibited in their ability to incorporate uridine.

In addition we have carried out preliminary studies on the effect of lomofungin on the incorporation of

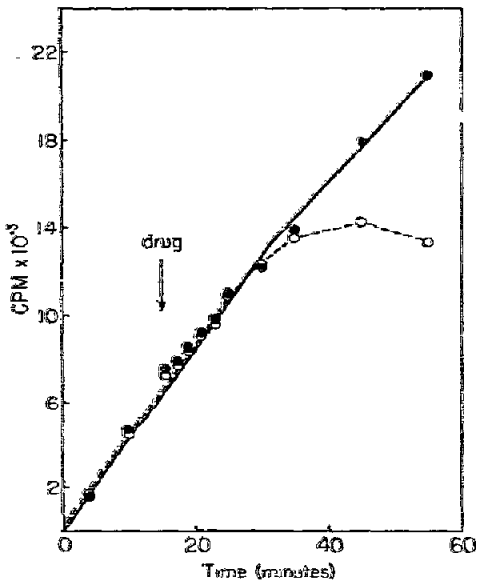

Fig. 2. Uptake of leucine by yeast spheroplasts and the effect of lomofungin. Conditions for preparation of spheroplasts are described under fig. 1. Samples (10 ml) were mixed with ^{3}H-labelled leucine (2 μCi) and cold leucine (10 μg). Incorporations with or without added lomofungin were carried out as under fig. 1 and samples (0.5 ml) removed into 10% TCA (0.5 ml) containing 1% casamino acids. Samples were heated at 90–95° for 15 min before processing and counting as described in Materials and methods. (•—•—•) = Control; (o--o--o) = sample with lomofungin added at the time indicated.

adenine into DNA. Our results indicate that DNA synthesis is rapidly inhibited by lomofungin (5 μg/ml) with kinetics similar to those for inhibition of RNA synthesis.

Since lomofungin is such a potent inhibitor of nucleic acid synthesis in both yeast cells and spheroplasts we have investigated the effect of the drug on the synthesis of protein by the latter. Spheroplasts were incubated with ^{3}H-labelled leucine, and samples removed into TCA at suitable time intervals. As for RNA synthesis, the incubation was continued for 15 min before drug addition, to establish linear incorporation conditions for the labelled amino acid. The result of this experiment is illustrated in fig. 2. In contrast to the results for uridine incorporation into RNA, the synthesis of protein, as measured by leucine uptake into hot TCA-precipitable material, is unaffected by lomofungin for approx. 20 min after addition of the drug. After this time the incorporation of leucine becomes inhibited and 30 min after drug addition there is little or no protein synthesis. This experiment has been repeated measuring uptake of ^{14}C-labelled methionine to study protein synthesis – the same result as for leucine was obtained. The effects of lomofungin on RNA and protein synthesis have also been examined in a double-labelling experiment using ^{3}H-labelled uridine and ^{14}C-labelled methionine. Results were identical to those shown in figs. 1 and 2.

Our results show, therefore, that while lomofungin inhibits total RNA synthesis in yeast spheroplasts almost immediately upon addition, it allows protein synthesis to proceed at a normal rate for a considerable length of time. Clearly, the drug does not inhibit protein synthesis directly and its effect on this process must be secondary. Because of this observation polyribosome profiles from yeast spheroplasts have been examined various times after addition of the drug. Profiles of both a control sample and a sample inhibited with lomofungin are identical (fig. 3a). Both samples were incubated for 5 min after either DMSO or drug addition, respectively. There is a small peak corresponding to 80 S ribosomes and the polyribosomes are clearly defined as peaks carrying presumably, 2, 3, 4, 5, 6, and possibly 7 and 8 monomeric 80 S ribosomes linked to messenger RNA. After 45 min incubation (fig. 3e) the control sample still retains much of its polyribosome content although the 80 S ribosome peak is more pronounced than in fig. 3a. However, spheroplasts, analyzed 45 min after lomofungin addition (fig. 3d) contain few polyribosomes and there is a massive peak of 80 S monomers. From this result it is clear that lomofungin induces ribosomes to 'run off' from messenger RNA. This is a progressive effect as shown by figs. 3b and 3c. Fifteen min after drug addition (fig. 3b) run off has commenced and by 30 min (fig. 3c) the polyribosome profile is disappearing rapidly to be replaced by a large peak of 80 S ribosomes.

The data obtained suggest that in *S. cerevisiae* lomofungin acts primarily as an inhibitor of nucleic acid synthesis, although we cannot as yet distinguish between effects on DNA or RNA production. It should be noted also that since under the growth conditions used in the present work mitochondrial function will be minimal, we cannot say whether this

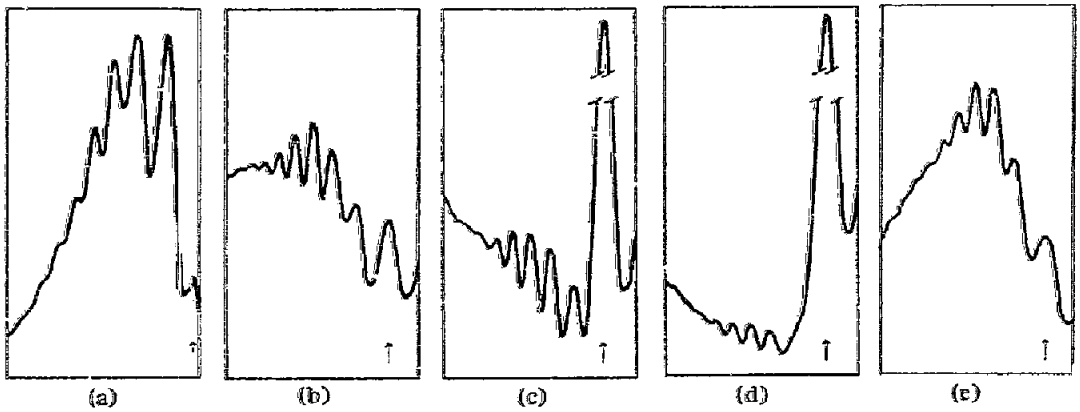

Fig. 3. Polyribosome profiles of yeast spheroplasts and the effect of incubation with lomofungin. Conditions for preparation of spheroplasts are described under fig. 1. Samples (10 ml) were incubated at 30° for the times indicated in the absence or presence of lomofungin (5 μg/ml). Protein synthesis was stopped by addition of cycloheximide (100 μg/ml) to 'freeze' polyribosomes and cultures were poured onto frozen M sorbitol (10 ml). Spheroplasts were collected by centrifugation and lysed and analyzed on sucrose gradients as described in the Materials and methods section. The tracings are plotted in arbitrary units and are meant to provide only a comparison of polyribosome profiles. In all cases the position of the 80 S ribosome peak is marked with an arrow and the left hand side of each tracing represents the bottom of the gradient. (a) Control sample and sample inhibited with lomofungin. Incubation for 5 min. These two tracings were identical and only one is depicted. (b) Sample incubated with lomofungin for 15 min. (c) Sample incubated with lomofungin for 30 min. (d) Sample incubated with lomofungin for 45 min. (e) Control sample incubated for 45 min.

cellular organelle could be affected by the drug.

Lomofungin primarily affects nucleic acid synthesis and an inhibition of protein synthesis follows only secondarily. Hence, we can determine approximately the decay of protein synthetic capacity which follows blockade of nucleic acid synthesis by the drug. This is important since, hitherto, *S. cerevisiae* has proved refractory to several inhibitors commonly used in other systems. Thus, although such drugs as rifamycin in bacteria and actinomycin D in higher eukaryotes have provided information on mRNA turnover in these organisms, the compounds fail to inhibit *S. cerevisiae*. The decline in incorporation of ^{3}H-labelled leucine into protein (fig. 2) and polyribosome run off (fig. 3) which both result from lomofungin inhibition can be used to estimate the decay in protein synthetic capacity in yeast spheroplasts when RNA synthesis is inhibited by the drug. In both cases decay occurs with a half life of 20–25 min, a figure which compares favorably with the time of 23 min determined by Hutchison et al. [6] in a mutant of *S. cerevisiae* temperature-sensitive for RNA synthesis. The half life of mRNA in bacteria approximates 3 min [7] while in higher organisms it may be as much as 2–3 days [8]. For our experiments it should be emphasized that the time of 20–25 min calculated above does not necessarily represent the actual half life of mRNA since other factors may be involved in the decay of protein synthetic capacity after inhibition of RNA synthesis by lomofungin.

Acknowledgements

We wish to thank the Medical Research Council of Great Britain, the National Institutes of Health and the Upjohn Company for financial support.

References

[1] C.D. Tipton and K.L. Rinehart, Jr., J. Am. Chem. Soc. 92 (1970) 1425.
[2] D. Gottlieb and G. Nicolas, Appl. Microbiol. 18 (1969) 35.
[3] H.T. Hutchison and L.H. Hartwell, J. Bacteriol. 94 (1967) 1697.
[4] S.A. Udem and J.R. Warner, J. Mol. Biol. 65 (1972) 227.
[5] S.A. Udem, K. Kaufman and J.R. Warner, J. Bacteriol. 105 (1971) 101.
[6] H.T. Hutchison, L.H. Hartwell and C.S. McLaughlin, J. Bacteriol. 99 (1969) 807.
[7] M. Schaechter, E.P. Previc and M.E. Gillespie, J. Mol. Biol. 12 (1965) 119.
[8] W. Murphy and G. Attardi, Proc. Natl. Acad. Sci. U.S. 70 (1973) 115.