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Inhibition of Influenza Virus Replication by α-Amanitin: Mode of Action

(chick embryo fibroblast/nucleus/polymerase II/parainfluenza virus)

B. W. J. MAHY, N. D. HASTIE, AND SYLVIA J. ARMSTRONG

Department of Pathology, University of Cambridge, England

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ABSTRACT **The replication of influenza virus in chick embryo fibroblast cells is inhibited by α-amanitin added during the first 2 hr of infection at concentrations similar to those required to inhibit cellular DNA-dependent RNA polymerase form II *in vivo*. Of two periods of increased RNA synthesis observed in cells infected with influenza virus, only the first, occurring from 0 to 2 hr after infection, is sensitive to α-amanitin. During this early period, there is a stimulation of the activity of DNA-dependent RNA polymerase II of nuclei isolated from infected cells. The data suggest that DNA transcription mediated by polymerase II is essential for influenza virus replication.**

The replication of influenza virus is sensitive in its early stages to treatment of the host cells with actinomycin D, mitomycin C, ultraviolet light, and other inhibitors of DNA function (1–5). The only other RNA viruses showing such dependence on functional DNA are the RNA tumour viruses which, unlike influenza (5–7), also require DNA synthesis after infection (8). These observations led to the hypothesis that the participation of host cellular DNA is essential for replication of influenza virus (9). Alternatively, it was proposed that actinomycin D and other drugs that bind to DNA inhibit replication of influenza virus in a nonspecific way, by degrading the infecting viral RNA molecules in the cell nucleus (3, 10), or by disorganization of the nucleolus (11). Support for the hypothesis that host cellular DNA is involved in influenza virus replication was provided by experiments showing that the virus stimulates cellular DNA-dependent RNA polymerase (EC 2.7.7.6) activity early in the infectious cycle (12, 13). Recently, it has been demonstrated in a number of eukaryotic cells that there are two major forms of DNA-dependent RNA polymerase, one (form I) situated in the nucleolus and involved in ribosomal RNA production, the other (form II) in the nucleoplasm and involved in synthesis of messenger RNA (14, 15). These two enzyme activities can be distinguished in whole nuclei by an *in vitro* assay with the drug α-amanitin (16), which inhibits DNA transcription by binding to form II polymerase without affecting form I polymerase activity (17–20). Using this drug, we have measured the relative activities of the two polymerases in cells infected with influenza virus; our data suggest that DNA-dependent RNA polymerase form II activity is essential during the early stages of virus replication.

MATERIALS AND METHODS

α-Amanitin was obtained from Boehringer Ingelheim Ltd., Isleworth, Middlesex, England, unlabeled nucleoside triphosphates from C. F. Boehringer and Sohne, Mannheim, Germany, and ^{3}H-labeled compounds from The Radiochemical Centre, Amersham, England.

Influenza A virus (fowl plague, Rostock strain) was grown in chick embryo fibroblast cultures as described (12). The cells were maintained either in 15-cm glass petri dishes, for enzyme studies, or in plastic "repli" dishes on 16 × 18-mm pieces of Melinex film for virus growth studies. Nuclei were purified by a modification of the Chauveau method (21) as described by Lawson *et al.* (22).

Enzyme Assays. The assay system used for RNA polymerase I and II activity was essentially as described by Novello and Stirpe (18). The reaction mixture for polymerase I contained in 0.6 ml: 50 μmol Tris·HCl(pH 8.0), 2 μmol $MgCl_2$, 7 μmol 2-mercaptoethanol, 3 μmol NaF, 0.06 μmol of each of ATP, CTP, and GTP, 2.5 μCi [^{3}H]UTP (3.0 or 10.5 Ci/mmol), 100 μg of calf thymus DNA, and about 200 μg of nuclear protein. The reaction mixture for polymerase II was identical except that $MgCl_2$ was replaced with $MnCl_2$, and 140 μmol of $(NH_4)_2SO_4$ were present in addition. Where indicated, 10 μg of actinomycin D or 2.5 μg of α-amanitin was added to the reaction mixture in a volume of 20 μl. Reaction mixtures were incubated for 15 min at 37° before incorporation of [^{3}H]UMP into acid-insoluble material was determined as described (23).

RNA polymerase I activity was calculated as total incorporation (cpm/mg of protein) minus incorporation in the presence of 10 μg of actinomycin D. This was necessary since actinomycin D-resistant RNA-dependent RNA polymerase activity, which is induced in nuclei of infected cells at about 2 hr after infection, interferes with assays for cellular polymerase performed in low salt, Mg^{++} conditions (13). RNA polymerase II activity was calculated as total incorporation (cpm/mg of protein) minus incorporation in the presence of 2.5 μg of α-amanitin (18).

RNA Synthesis in Chick Embryo Fibroblasts. The growth medium [Eagles minimal essential medium (MEM) containing 10% calf serum] of confluent monolayer cultures of chick embryo fibroblast cells grown on 16 × 18-mm Melinex pieces (2×10^6 cells per piece) was replaced with 1 ml of maintenance medium containing 3% calf serum. At intervals, 10 μCi of [^{3}H]uridine (27.6 Ci/mmol) was added to the medium for 15 min after which cultures were washed successively with phosphate-buffered saline (pH 7.4), ice-cold 0.5 N trichloroacetic acid (twice), and acetone (twice). The preparations were dried, then immersed in a toluene-based scintillation fluid for determination of radioactivity.

Virus Growth Studies. Replicate chick embryo fibroblast cultures were washed and exposed to influenza virus (input multiplicity of 15–20 plaque forming units per cell) for 30 min

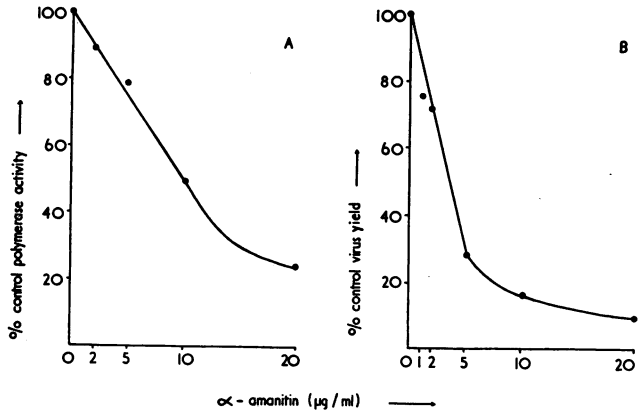

FIG. 1. (*A*) Effect of α-amanitin during growth on RNA polymerase II activity in normal chick embryo fibroblast cells. The growth medium of confluent monolayer cultures of chick embryo fibroblast cells grown in 15-cm glass petri dishes was replaced with medium containing 3% calf serum and various concentrations of α-amanitin up to 20 μg/ml. After incubation for 1 hr at 37°, the cells were washed extensively in 0.9% saline, after which the nuclei were purified and assayed for RNA polymerase II activity in the presence or absence of 2.5 μg of α-amanitin as described in *Methods*. The results are plotted as a percentage of RNA polymerase II activity (total incorporation minus incorporation in the presence of 2.5 μg of α-amanitin) of untreated control cultures (85,380 cpm/mg of protein per 15 min at 37°).

(*B*) Effect of α-amanitin on influenza virus growth. Replicate chick embryo fibroblast cultures were washed and exposed to influenza virus (input multiplicity of 15–20 plaque forming units per cell) for 30 min at 37°. Unadsorbed virus was removed, and the cells were washed twice before addition of fresh medium at 37° containing various α-amanitin concentrations. After 24-hr incubation at 37°, the amount of viral hemagglutinin released into the medium was determined. Each point is the mean of duplicate determinations on separate cultures.

at 37°. Unadsorbed virus was removed and the cells were washed twice before addition of fresh maintenance medium at 37°. After 24-hr incubation at 37°, the amount of viral hemagglutinin released into the medium was determined.

RESULTS

The effect of various doses of α-amanitin on the yield of influenza virus from infected chick embryo fibroblast cultures is shown in Fig. 1. More than 90% inhibition of virus growth occurred at a drug concentration of 20 μg/ml of medium. Plaque assays showed that virus yields obtained in the presence of α-amanitin had normal infectivity to hemagglutinin ratios; this agrees with a previous report that cell-associated viral hemagglutinin and infectivity were inhibited equally by α-amanitin (26). In a parallel experiment, uninfected cells were treated with the same concentrations of α-amanitin, and after 1 hr incubation at 37°, the cells were washed extensively in 0.9% saline, after which the nuclei were purified and assayed for RNA polymerase II activity. Increasing doses of α-amanitin caused a progressive inhibition of RNA polymerase II activity (Fig. 1). These results indicate that a close correlation exists between inhibition of virus yield and of RNA polymerase II activity in the α-amanitin-treated cells.

The replication of influenza virus was previously shown to be sensitive to actinomycin D only if the drug was present during the first 2 hr after infection (1, 4). Since actinomycin D inhibits RNA synthesis by binding directly to DNA (9), whereas α-amanitin binds not to the DNA template but to the polymerase itself (17–20) it was of interest to determine when influenza replication was most sensitive to α-amanitin. As shown in Fig. 2, inhibition of influenza virus yield occurred when α-amanitin (20 μg/ml) was added to the medium 1 hr before, or up to 2 hr after, infection; if added 3 hr or later after infection the virus yield was normal. Thus, both actinomycin D and α-amanitin inhibit an event occurring within the first 2 hr after infection with influenza virus.

RNA synthesis in chick fibroblast cells infected with the fowl plague strain of influenza virus is characterized by two periods of increased incorporation of [^{3}H]uridine into RNA, the first occurring at 0.5–1 hr, the second at 2–4 hr after infection (12). To determine the effect of α-amanitin on this virus-induced RNA synthesis, we added the drug at various times from 1 hr before to 3 hr after infection. 1 hr after addition of α-amanitin, infected and uninfected cells were pulse-labeled for 15 min with [^{3}H]uridine before determination of the amount of incorporation of radioactivity into acid-insoluble material. At the same time, another set of infected and uninfected cells were pulse-labeled with [^{3}H]uridine without prior treatment with α-amanitin. The results are expressed as the percentage incorporation of label into infected compared to uninfected cells in each case (Fig. 3). The presence of α-amanitin completely inhibited the early phase of increased virus-induced RNA synthesis occurring between 0 and 1.5 hr after infection, but did not alter the relative rate of RNA synthesis in infected cells between 2 and 4 hr after infection. This result was obtained reproducibly in several separate experiments.

RNA polymerase I and II activities were measured in nuclei of cells infected with influenza virus for various periods up to 6 hr after infection, and compared with the activities in uninfected cell nuclei taken at each point (Fig. 4). RNA polymerase I activity decreased between 1 and 2 hr after infection and was less than 20% of the activity in uninfected cells by

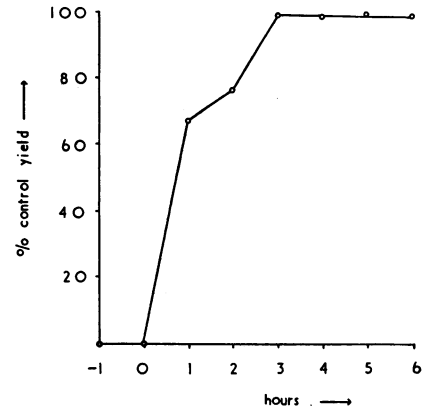

FIG. 2. Effect of time of addition of α-amanitin on influenza virus growth. Cells were infected as described in the legend to Fig. 1, and at various times from 1 hr before to 6 hr after infection the medium was supplemented with 20 μg of α-amanitin per ml. After 24 hr, the amount of viral hemagglutinin released into the medium was determined, and the results were expressed as percentage of virus yield in untreated cultures. Each point is the mean of triplicate determinations on separate cultures.

3–4 hr. However, polymerase II activity in infected cells increased to a maximum, at 1.5 hr after infection, of 70% above the activity in uninfected cells.

A similar experiment was performed in which RNA polymerase II activities were measured in cells infected with Sendai virus. This was of interest since the replication of this parainfluenza virus is insensitive to α-amanitin at doses that completely inhibit the replication of influenza virus. As shown in Fig. 4, no stimulation of nuclear RNA polymerase II activity was observed in infected cells during the latent period of Sendai virus replication.

DISCUSSION

α-Amanitin, a bicyclic octapeptide from the toadstool, *Amanita phalloides* (16), inhibits DNA transcription by binding specifically to RNA polymerase form II of eukaryotic organisms (17–20). Of a number of RNA viruses that have been tested (24), only Rous sarcoma (25) and influenza (26) viruses are inhibited by this drug. We have found that replication of influenza virus is inhibited only if α-amanitin is present during the first 2 hr after infection, at concentrations similar to those required to inhibit RNA polymerase form II in the host cells. During this early period, there is a stimulation of RNA synthesis in infected cells that is abolished by α-amanitin, and DNA-dependent RNA polymerase form II activity of nuclei isolated from infected cells is enhanced. These results complement previous evidence, based on studies with actinomycin D (9), that host-cell DNA-directed RNA synthesis is essential during the early stages of influenza virus replication.

From 2 to 4 hr after infection, a second period of increased RNA synthesis is observed in infected cells, and this is insensitive to α-amanitin. It probably results from RNA-dependent RNA polymerase activity, since both the RNA-dependent RNA transcriptase found in influenza virions (27) and the RNA-dependent RNA polymerase induced in influenza-infected cells (26) have been shown to be unaffected by α-amanitin. It is interesting to note that at this time there was a sharp decline in DNA-dependent RNA polymerase form I activity in influenza virus-infected cells (Fig. 4), since a cut-off in host-cell RNA polymerase activity is a feature of many viral infections (28).

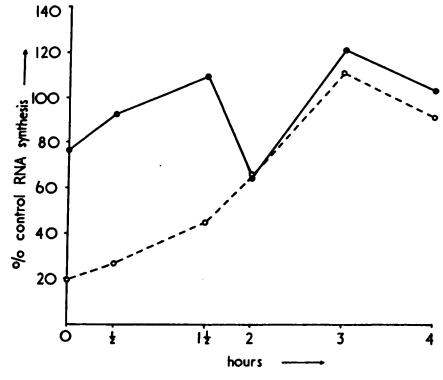

FIG. 3. Effect of α-amanitin on RNA synthesis in influenza virus-infected cells. Triplicate cultures, either uninfected or infected as described in the legend to Fig. 1, were pulse-labeled with [^{3}H]uridine for 15 min at various times as described in *Methods*. Another set of cultures was treated in the same way, except that α-amanitin (20 μg/ml) was added for 1 hr before each uridine pulse. For both sets of cultures, acid-insoluble radioactivity incorporated into uninfected cells during the 15-min pulse was about 5000 cpm. The results are expressed as percentage incorporation of [^{3}H]uridine into acid-insoluble material of infected compared to uninfected cultures. ●——●, untreated; O- - - O, α-amanitin-treated.

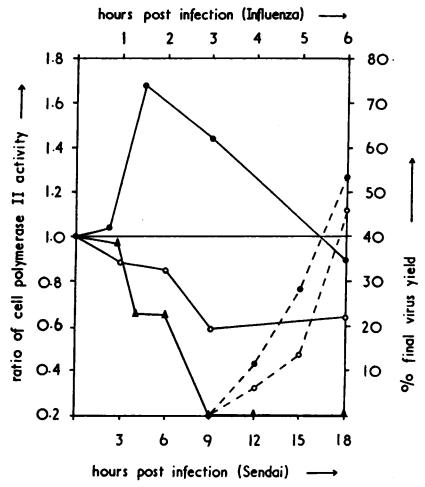

FIG. 4. DNA-dependent RNA polymerase activities of cells infected with influenza or Sendai virus for various periods of time. Replicate cultures of chick embryo fibroblast cells, either uninfected or infected with influenza or Sendai viruses (input multiplicity of 15–20 plaque forming units per cell) as described (12, 33) were harvested at different times postinfection, and nuclei were purified and assayed for RNA polymerase I and II activity as described in *Methods*. Results are plotted as the ratio of activity in infected compared to uninfected cells at each time point. Influenza and Sendai viral growth was followed by determination of the amount of hemagglutinin released into the culture medium (12), and the results were expressed as a percentage of the final yield in parallel cultures incubated for 24 hr. Each point is the mean of duplicate determinations.

Influenza virus-infected cells: ▲——▲, RNA polymerase I; ●——●, RNA polymerase II; ●- - - ●, released hemagglutinin. Sendai virus-infected cells: O——O, RNA polymerase II; O- - - O, released hemagglutinin.

Rott and Scholtissek (26) reported inhibition of influenza virus replication by α-amanitin under conditions in which there was little inhibition of total cellular RNA synthesis. We have also found that cellular RNA synthesis as measured by ^{3}[H]uridine incorporation into acid-insoluble material is not significantly inhibited for 2–3 hr after addition of α-amanitin (Armstrong, Hastie, and Mahy, manuscript in preparation), but, as shown in Fig. 1, the drug, when added to cells, causes a rapid inhibition of nuclear RNA polymerase II activity. This suggests that polymerase II activity contributes little to the overall rate of cellular RNA synthesis as measured by [^{3}H]uridine incorporation.

The relative activities of RNA polymerases I and II observed *in vitro* may not be simply related to their relative rates of RNA synthesis *in vivo*. For example, assays performed at high ionic strength may unmask not only DNA template but also endogenous RNA polymerase activity normally bound within chromatin (29). Experiments are in progress to determine whether the increased transcriptional activity in influenza virus-infected cells is due to alteration in template

availability, or to an increase in the amount or catalytic ability of RNA polymerase, or both.

Recent evidence suggests that the RNA polymerase activity that is sensitive to α-amanitin is located within the nucleoplasm and is reponsible for the bulk of messenger RNA synthesis, (14, 15, 29, 30). Thus, our results would suggest that a species of host cell-coded messenger RNA is necessary for replication of influenza virus. Previous studies have indicated that the influenza genome may not contain sufficient information to code for all the structural and nonstructural polypeptides associated with viral growth (31, 32). At least a part of this information may be provided by the host cell.

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1. Barry, R. D., Ives, D. R. & Cruickshank, J. G. (1962) "Participation of deoxyribonucleic acid in the multiplication of influenza virus," *Nature* **194**, 1139–1140.
2. Barry, R. D. (1964) "The effects of actinomycin D and ultraviolet irradiation on the production of fowl plague virus," *Virology* **24**, 563–569.
3. Rott, R., Saber, S. & Scholtissek, C. (1965) "Effect on myxovirus of mitomycin C, actinomycin D, and pretreatment of the host cell with ultraviolet light," *Nature* **205**, 1187–1190.
4. White, D. O., Day, H. M., Batchelder, E. J., Cheyne, I. M. & Wansbrough, A. J. (1965) "Delay in the multiplication of influenza virus," *Virology* **25**, 289–302.
5. Nayak, D. P. & Rasmussen, A. F. (1966) "Influence of mitomycin C on the replication of influenza viruses," *Virology* **30**, 673–683.
6. Scholtissek, C. & Rott, R. (1961) "Connection between the synthesis of ribonucleic acid and protein in the growth of a virus of the influenza group (fowl plague virus)," *Z. Naturforsch. B*, **16**, 663–673.
7. Buthala, D. A. (1964) "Cell culture studies on antiviral agents I, Action of cytosine arabinoside and some comparisons with 5-iodo-2-deoxyuridine," *Proc. Soc. Exp. Biol.* **115**, 69–77.
8. Bader, J. P. (1965) "The requirement for DNA synthesis in the growth of Rous sarcoma and Rous-associated viruses," *Virology* **26**, 253–261.
9. Barry, R. D. (1964) in "Effects of inhibitors of nucleic acid synthesis of the production of myxoviruses," *Ciba Foundation Symposium on Cellular Biology of Myxovirus Infections*, eds. Wolstenholme, G. E. W. & Knight, J. (Churchill, London), pp. 51–71.
10. Ghendon, Yu., Ginsburg, V. P., Soloviev, G. Ya. & Markushin, S. G. (1970) "The fate of influenza virus RNA in cells treated with ultraviolet rays," *J. Gen. Virol.* **6**, 249–255.
11. Dimmock, N. J. (1969) "New virus-specific antigens in cells infected with influenza virus," *Virology* **39**, 224–234.
12. Borland, R. & Mahy, B. W. J. (1968) "Deoxyribonucleic acid-dependent ribonucleic acid polymerase activity in cells infected with influenza virus," *J. Virol.* **2**, 33–39.
13. Mahy, B. W. J. (1970) in "The replication of fowl plague virus RNA," *The Biology of Large RNA Viruses*, eds. Barry, R. D. & Mahy, B. W. J. (Academic Press, New York), pp. 392–415.
14. Roeder, R. G. & Rutter, W. J. (1969) "Multiple forms of DNA-dependent RNA polymerase in eukaryotic organisms," *Nature* **224**, 234–237.
15. Roeder, R. G. & Rutter, W. J. (1970) "Specific nucleolar and nucleoplasmic RNA polymerase," *Proc. Nat. Acad. Sci. USA* **65**, 675–682.
16. Fiume, L. & Wieland, Th. (1970) "Amanitins. Chemistry and action," *FEBS Lett.* **8**, 1–5.
17. Kedinger, C., Gniazdowski, M., Mandel, J. L., Gissinger, F. & Chambon, P. (1970) "α-Amanitin: a specific inhibitor of one of two DNA-dependent RNA polymerase activities from calf thymus," *Biochem. Biophys. Res. Commun.* **38**, 165–171.
18. Novello, F. & Stirpe, F. (1970) "Simultaneous assay of RNA polymerase I and II in nuclei isolated from resting and growing rat liver with the use of α-amanitin," *FEBS Lett.* **8**, 57–60.
19. Chesterton, C. J. & Butterworth, P. H. W. (1971) "Studies on the origin of the form Ib mammalian DNA-dependent RNA polymerase," *FEBS Lett.* **13**, 275–278.
20. Lindell, T. J., Weinberg, F., Morris, P. W., Roeder, R. G. & Rutter, W. J. (1970) "Specific inhibition of RNA polymerase II by α-amanitin," *Science* **170**, 447–449.
21. Chauveau, J., Moule, Y. & Rouiller, Ch. (1956) "Isolation of pure and unaltered liver nuclei. Morphology and biochemical composition," *Exp. Cell Res.* **11**, 317–321.
22. Lawson, D. E. M., Wilson, P. W., Barker, D. C. & Kodicek, E. (1969) "Isolation of chick intestinal nuclei," *Biochem. J.* **115**, 263–268.
23. Mahy, B. W. J. & Bromley, P. A. (1970) "*In vitro* product of a ribonucleic acid polymerase induced by influenza virus," *J. Virol.* **6**, 259–268.
24. Fiume, L., La Placa, M. & Portolani, M. (1966) "Ricerche sul meccanismo dell'azione citopatogena dell α-amanitin," *Sperimentale* **116**, 15–25.
25. Zanetti, M., Foa, L., Costanzo, F. & La Placa, M. (1971) "Specific inhibition of Rous sarcoma virus by α-amanitin," *Arch. Gesamte Virusforsch.* **34**, 255–260.
26. Rott, R. & Scholtissek, C. (1970) "Specific inhibition of influenza replication by α-amanitin," *Nature* **228**, 56.
27. Penhoet, E., Miller, H., Doyle, M. & Blatti, S. (1971) "RNA-dependent RNA polymerase activity in influenza virions," *Proc. Nat. Acad. Sci. USA* **68**, 1369–1371.
28. Martin, E. M. & Kerr, I. M. (1968) in "Virus-induced changes in host-cell macromolecular synthesis," *The Molecular Biology of Viruses*, eds. L. V. Crawford & Stoker, M. G. P. (Cambridge University Press), pp 15–46.
29. Butterworth, P. H. W., Cox, R. F. & Chesterton, C. J. (1971) "Transcription of mammalian chromatin by mammalian DNA-dependent RNA polymerases," *Eur. J. Biochem.* **23**, 229–241.
30. Zylber, E. A. & Penman, S. (1971) "Products of RNA polymerases in HeLa cell nuclei," *Proc. Nat. Acad. Sci. USA* **68**, 2861–2865.
31. Compans, R. W., Klenk, H. D., Caliguiri, L. A. & Choppin, P. W. (1970) "Influenza virus proteins: 1. Analysis of polypeptides of the virion and identification of spike glycoproteins," *Virology* **42**, 880–889.
32. Schulze, I. T. (1970) "The structure of influenza virus. I. The polypeptides of the virion," *Virology* **42**, 890–904.
33. Mahy, B. W. J., Hutchinson, J. E. & Barry, R. D. (1970) "Ribonucleic acid polymerase induced in cells infected with Sendai virus," *J. Virol.* **5**, 663–671.