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The Antibacterial Action of Tinopal AN

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The bactericidal activity of Tinopal AN [1,1-bis(3,*N*-5-dimethyl-benzoxazol-2-yl)-methine *p*-toluene sulphonate] was shown to be due to a mechanism entirely independent of its inhibitory effects upon NADH dehydrogenase which were reported previously. Whereas the compound had no significant effect upon DNA synthesis in *Escherichia coli* D22, RNA and protein synthesis were immediately and markedly inhibited. In confirmation, Tinopal AN caused an immediate cessation in inducible β-galactosidase synthesis in the same organism. An *in vitro* assay of the transcription of calf-thymus DNA by purified *E. coli* RNA polymerase showed that this process was inhibited by Tinopal AN.

INTRODUCTION

Tinopal AN [1,1-bis(3,*N*-5-dimethyl-benzoxazol-2-yl)-methine *p*-toluene sulphonate] is a cationic benzoxazole compound which has often been used as a fluorescent optical brightener in UV-light microscopy (Paton & Jones, 1971, 1973). It was later shown by Gonzalez-Lopez *et al.* (1981) that low concentrations of the compound were rapidly and selectively lethal to certain bacteria, in particular towards phytopathogenic but not towards saprophytic species of *Pseudomonas*, *Xanthomonas* and *Corynebacterium*.

Studies using another Tinopal AN-sensitive, but biochemically better-characterized respiratory bacterium, *Paracoccus denitrificans*, were carried out to determine the mode of antibacterial action on such sensitive cells. Low concentrations of Tinopal AN markedly inhibited respiration in the NADH dehydrogenase region of the respiratory chain in this organism (Phillips & Kell, 1981). Further experiments (Phillips & Kell, 1982) were carried out to determine whether Tinopal AN acted at the same site as other segment I inhibitors such as rotenone, amytal and piericidin A, which were all shown to bind to the same polypeptide (Horgan *et al.*, 1968), namely the iron–sulphur centre N-2 (Meijer *et al.*, 1978). The results showed that Tinopal AN did not share the same specific binding site as rotenone (and also presumably amytal and piericidin A), and hence provided the first example of an inhibitor of the NADH dehydrogenase region of the respiratory chain whose binding site was not associated with iron–sulphur centre N-2 (Phillips & Kell, 1982). Maguire *et al.* (1983) subsequently reported that the respiratory block by Tinopal AN occurred on the oxidizing side of cluster N-2 in submitochondrial particles.

However, the growth of two obligately fermentative *Clostridium* species was found to be markedly sensitive to low concentrations of Tinopal AN; to account for these and other findings it was hypothesized (Phillips, 1983) that the lethal effect of Tinopal AN on cell growth was due to another mechanism which was distinct from the previously described respiratory inhibition. In this study we report further evidence for a non-respiratory site of lethal action in *Escherichia coli*; it was found that Tinopal AN acted as a potent inhibitor of DNA-dependent RNA synthesis both *in vivo* and *in vitro*.

Abbreviations: IPTG, isopropyl-β-D-thiogalactoside; ONPG, *o*-nitrophenylgalactoside.

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METHODS

Organisms and growth conditions. Paracoccus denitrificans NCIB 8944 was grown and maintained aerobically in succinate–nitrate medium at 30 °C (Burnell *et al.*, 1975; McCarthy *et al.*, 1981). *Escherichia coli* D22 (*envA1*) was kindly donated by Dr G. Fletcher, Department of Microbiology, University of Texas, Austin, Texas 78712, USA. It was an outer-membrane mutant containing less lipopolysaccharide than the wild-type, and had a greater sensitivity towards a wide range of antibiotics compared with the wild-type (Normark *et al.*, 1969; Grundström *et al.*, 1980). Unlike the wild-type it was also sensitive to Tinopal AN and so this mutant strain was used in all the radioisotopic experiments.

Growth experiments. Cells from an overnight culture of *P. denitrificans* grown aerobically at 30 °C in the medium cited above were used as an inoculum for fresh medium and incubated further at 30 °C. Aerobic growth was assessed at 550 nm using a Bausch & Lomb Spectronic 70 spectrophotometer. Addition of Tinopal AN or 2·0% (v/v) ethanol (control) was made when the early exponential phase of growth (about 3×10^7 cells ml^{-1}) was reached. A similar experiment was carried out using *E. coli* D22 (*envA1*) grown aerobically at 37 °C in 250 ml tryptone soya broth which contained ($g\ l^{-1}$): tryptone, 1·7; soyatone, 0·3; NaCl, 0·5 and K_2HPO_4, 0·25. Aerobic growth was assessed at 450 nm.

Respiration measurements. Escherichia coli D22 (*envA1*) was grown aerobically at 37 °C in 250 ml minimal medium (M9) which contained ($g\ l^{-1}$): $NaHPO_4$ (anhydrous), 6·0; KH_2PO_4 (anhydrous), 3·0; NaCl, 0·5; NH_4Cl, 1·0; $CaCl_2.2H_2O$, 0·01; $MgSO_4.7H_2O$, 0·25; sodium succinate, 10·0; L-proline, 0·2; L-tryptophan, 0·2 and L-histidine, 0·2. Mid-exponential phase cells were harvested, washed twice in 250 ml 0·1 M-sodium phosphate buffer, pH 7·3 and resuspended in the same buffer to a final concentration of 20 mg dry wt ml^{-1}.

Respiration was monitored by adding 1·5 mg dry wt cells to a 3 ml reaction mixture in a Clark-type oxygen electrode vessel containing 0·1 M-sodium phosphate buffer, pH 7·3 and 20 mM-sodium succinate at 30 °C (Kell *et al.*, 1978). Tinopal AN dissolved in ethanol, or ethanol alone (control) was added and the respiration rates were monitored after each addition.

Radioisotopes. [*Me*-^{3}H]TTP, [2-^{14}C]uracil, L-[U-^{14}C]proline and [8-^{14}C]ATP were obtained from Amersham.

Uptake of thymidine. The non-thymine-requiring strain, *E. coli* D22 (*envA1*), was grown aerobically at 37 °C in 500 ml minimal medium (M9, described above) that also contained ($g\ l^{-1}$): deoxyadenosine, 0·2 (Gilbert *et al.*, 1980) and TTP, 0·004. Mid-exponential phase cells (about 5×10^7 cells ml^{-1}) were washed three times in 40 mM-Tris/HCl, pH 8·0, and resuspended in growth medium lacking deoxyadenosine, at 4 °C, to give a final cell concentration of 15 mg dry wt ml^{-1}. Cells (3 ml, 45 mg dry wt) from this suspension were added to fresh medium lacking deoxyadenosine and TTP to give a cell concentration of approximately 1·5 mg dry wt ml^{-1}; the suspension was preincubated for 5 min at 37 °C. At zero time, 0·24 μM-[*Me*-^{3}H]TTP (0·32 μCi $nmol^{-1}$; 11·84 kBq $nmol^{-1}$) was added.

Samples were removed at regular intervals and added to an equal volume (1 ml) of 10% (w/v) TCA containing 0·05% (w/v) thymidine and 0·2 M-KH_2PO_4 at 0 °C. After 90 min the amount of label taken up into the insoluble macromolecules was measured by filtering samples through glass fibre filters (Whatman GF/C, 2·5 cm diam.) previously soaked in 5% TCA. Filters were washed twice using 2×5 ml 5% TCA and dried with an IR lamp. The amount of radioactivity was measured in 3 ml scintillation fluid [toluene/Triton X-100 (2:1, v/v) containing 4 g 2,5-diphenyloxazole l^{-1}] using a Philips PW 4540 liquid scintillation analyser.

Uptake of uracil. Escherichia coli D22 (*envA1*) was cultured and harvested as described above in M9 medium lacking deoxyadenosine and TTP. Washed organisms were resuspended in fresh growth medium containing 10 μg uracil ml^{-1} to a cell concentration of 20 mg dry wt ml^{-1} at 4 °C; 3 ml cells (60 mg dry wt) were added to fresh medium containing 10 μg uracil ml^{-1} (1·7 mg dry wt cells ml^{-1}) and incubated for 5 min at 37 °C. At zero time 0·05 μCi (1·85 kBq) [2-^{14}C]uracil ml^{-1} (50 Ci mol^{-1}; 1·85 TBq mol^{-1}) was added, and the amount of radioactivity in TCA-insoluble material was determined at regular intervals as described above.

Uptake of proline. Escherichia coli D22 (*envA1*) was cultured in 500 ml M9 medium, washed and harvested as above, and resuspended in fresh growth medium containing 10 μg L-proline ml^{-1} at 4 °C to give 9 mg dry wt cells ml^{-1}. Cells (27 mg dry wt) were added to fresh medium lacking L-proline to give a cell concentration of 0·9 mg dry wt ml^{-1} and incubated aerobically for 5 min at 37 °C. At zero time, 0·033 μCi (1·221 kBq) L-[U-^{14}C]proline ml^{-1} (33·3 μCi $mmol^{-1}$; 1·232 MBq $mmol^{-1}$) was added. Samples were removed and the amount of radioactivity in TCA-insoluble material was determined as described above.

Induction and assay of β-galactosidase activity. Escherichia coli D22 (*envA1*) was grown aerobically at 37 °C in M9 minimal medium containing 1% (w/v) glycerol as carbon and energy source. Early-exponential phase cells were washed and resuspended to a concentration of 0·5 mg dry wt ml^{-1} in fresh medium at 37 °C. Samples (0·5 ml) were removed at regular intervals and assayed for β-galactosidase both before and after the addition of 3·5 mM isopropyl-β-D-thiogalactoside (IPTG). Samples were added to an equal volume (0·5 ml) of 0·2 M-sodium phosphate buffer, pH 7·0 containing one drop of toluene and mixed thoroughly. Suspensions were incubated for 40 min at 37 °C or until the toluene had evaporated. A 0·2 ml volume of 13·5 mM-*o*-nitrophenylgalactoside (ONPG) was then added. The time required for the development of a yellow colour was recorded and 0·5 ml 1 M-Na_2CO_3 was

added to stop the reaction. The A_{420} and A_{550} values were determined on a Bausch & Lomb Spectronic 70 spectrophotometer. The specific activity of enzyme was calculated from the relationship:

$$\text{Specific activity } |\Delta A_{420}\,\text{min}^{-1}\,(\text{mg dry wt})^{-1}| = \frac{A_{420} - 1{\cdot}75\,A_{550}}{\text{Time (min)} \times \text{mg dry wt}}$$

DNA transcription. Transcription of calf-thymus DNA (Boehringer Mannheim) was carried out at 37 °C according to the method of Burgess & Travers (1971) in a 1 ml reaction mixture which contained: Tris/HCl buffer, pH 7·9, 40 mM; $MgCl_2$, 10 mM; EDTA, 0·1 mM; DTT, 0·1 mM; KCl, 150 mM; K_2HPO_4, 0·4 mM; bovine serum albumin, 0·5 mg ml^{-1}; CTP, 0·15 mM; GTP, 0·15 mM; UTP, 0·15 mM; [8-^{14}C]ATP, 0·15 mM (1 μCi mol^{-1}; 37 kBq mol^{-1}); calf-thymus DNA, 0·15 mg ml^{-1}. At zero time, 1 unit of RNA polymerase (300 units mg^{-1}) from *E. coli* MRE 600 (Boehringer Mannheim) was added. Samples (0·1 ml), taken at regular intervals to assess the amount of radioactivity incorporated into TCA-insoluble material, were added to 0·5 ml 10% TCA at 0 °C. After 90 min at 0 °C, suspensions were filtered, washed, dried and counted for their radioactivity as described above.

Chemicals. Tinopal AN (2 mM in 10% ethanol) was kindly donated by Dr A. M. Paton, Division of Agricultural Bacteriology, School of Agriculture, University of Aberdeen, UK. Nalidixic acid, which was dissolved in alkaline solution, and IPTG, ONPG, chloramphenicol and DTT were obtained from Sigma.

RESULTS AND DISCUSSION

Effect of Tinopal AN on the growth kinetics of P. denitrificans

Previous studies revealed that the addition of rotenone (a respiratory inhibitor of the NADH dehydrogenase region of the electron transport chain) to exponentially growing *P. denitrificans* resulted in an immediate cessation of growth, followed after about 1 h by a recovery of growth (Meijer *et al.*, 1978). We have confirmed these findings (Phillips & Kell, 1981, 1982) both for rotenone, and also for antimycin A (Phillips, 1983), a segment II inhibitor. An immediate cessation of growth occurred when rotenone was added to give concentrations of 100 μM (300 nmol mg dry wt^{-1}) or 200 μM (600 nmol mg dry wt^{-1}); however, in both cases, growth resumed after about 1 h at rates similar to those attained in the absence of rotenone (Meijer *et al.*, 1978; Phillips & Kell, 1982). Similar effects were recently reported using the electron transport inhibitor myxothiazol (Thierbach & Reichenbach, 1983). Figure 1 shows the effect of the addition of 20 μM-Tinopal AN in a similar experiment; growth was immediately inhibited and did not recover. Lower concentrations of Tinopal AN (1, 5 and 10 μM) reduced the growth rates, which, it should be noted, did not subsequently recover (Fig. 1).

These results suggested either an inability of the cells to 'bypass' the Tinopal AN-sensitive site in the respiratory chain, or that, in addition to its ability to inhibit respiration in sensitive cells, the compound exerted its lethal effects by another mechanism. That Tinopal AN was bactericidal and not merely bacteriostatic, was shown previously (Gonzalez-Lopez *et al.*, 1981).

Effect of Tinopal AN on the respiration and growth of E. coli D22 (envA1)

When the effect of Tinopal AN on the respiration of *E. coli* D22 (*envA1*) was examined it was apparent that additions of up to 100 μM did not significantly affect the respiration rate of washed cell suspensions, as determined by the oxygen uptake assay (Fig. 2). However, the growth of the organism was markedly sensitive to the compound; growth of exponential-phase cells was immediately inhibited on addition of 20 μM-Tinopal AN (Fig. 3). In addition a 5 mm zone of inhibition occurred round a 1 mM-Tinopal AN filter-paper strip (Phillips, 1983). Hence these findings not only provided further evidence for a non-respiratory site of Tinopal AN toxicity, but also showed this strain to be an ideal test organism to distinguish any potentially lethal effects of Tinopal AN which were independent of a possible respiratory inhibition, because the respiration of *E. coli* D22 (*envA1*) was insensitive to this compound. The *envA1* strain was used in the following investigations because its growth was inhibited in the presence of 20 μM-Tinopal AN (Fig. 3).

Effect of Tinopal AN on DNA synthesis in E. coli D22 (envA1)

The synthesis of DNA in exponentially-growing cells was measured as the incorporation into TCA-insoluble material of [*Me*-^{3}H]TTP. Rapid uptake of the label occurred over the 60 min incubation period, corresponding to a rapid synthesis of DNA in these exponential phase cells

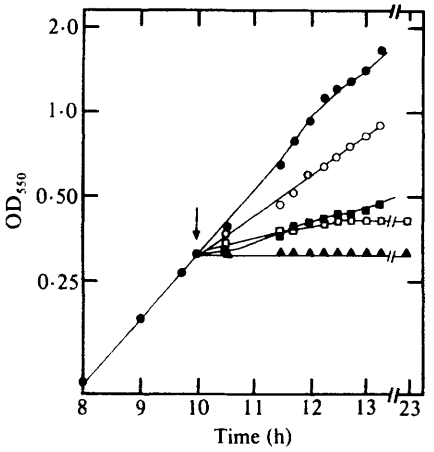

Fig. 1

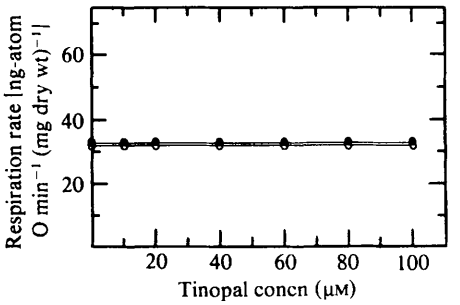

Fig. 2

Fig. 1. The inhibition of the growth of *P. denitrificans* by Tinopal AN. Aerobic growth in succinate–nitrate medium at 30 °C was measured at 550 nm. The arrow indicates addition to the culture of 1 μM (○), 5 μM (■), 10 μM (□) or 20 μM (▲) Tinopal AN. ●, Control culture.

Fig. 2. Effect of Tinopal AN on the respiration of *E. coli* D22 (*envA1*). The respiration of washed mid-exponential cells (1·5 mg dry wt) was monitored in a Clark-type oxygen electrode containing 0·1 M-sodium phosphate buffer, pH 7·3, plus 20 mM-sodium succinate at 30 °C (Kell *et al.*, 1978). Additions of Tinopal AN (●) or ethanol (control, ○) were made and the respiration was monitored.

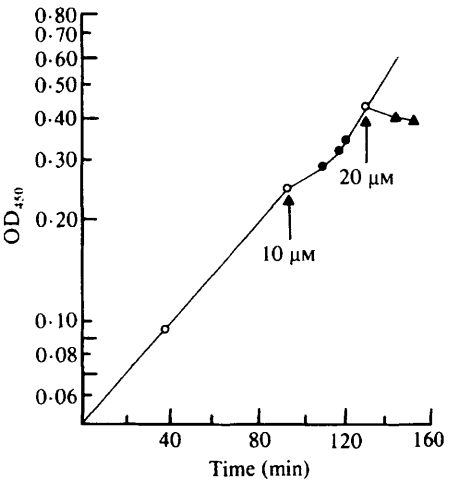

Fig. 3. The inhibition of the growth of *E. coli* D22 (*envA1*) by Tinopal AN. Aerobic growth in tryptone soya broth was measured at 450 nm. Arrows indicate addition to the culture of 10 μM (●) or 20 μM (▲) Tinopal AN. ○, Control.

(Fig. 4). Addition of 40 μg nalidixic acid ml^{-1}, a concentration which maximally inhibits DNA gyrase (Sanzey, 1979), caused a delayed inhibition of synthesis, which was unaffected for 20 min after the addition of the inhibitor to the culture. However, addition of 20 μM-Tinopal AN had no significant effect on the uptake and incorporation of the label (and hence DNA synthesis) within the experimental time period (Fig. 4). It was thus concluded that Tinopal AN did not significantly inhibit DNA synthesis in *E. coli* and that a possible action involving inhibition of DNA synthesis did not account for the growth sensitivity shown by this organism towards Tinopal AN.

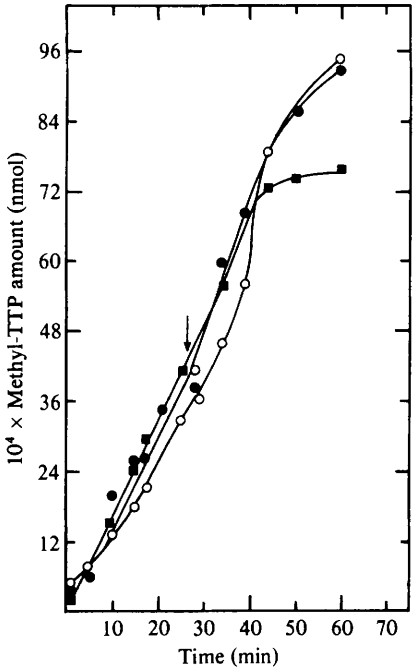

Fig. 4

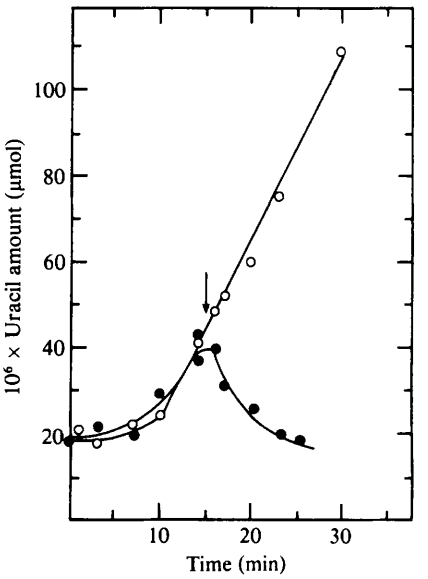

Fig. 5

Fig. 4. The effect of Tinopal AN on DNA synthesis in *E. coli* D22 (*envA1*). DNA synthesis was measured by uptake and incorporation of [*Me*-^{3}H]TTP by the culture. The arrow indicates the addition to the culture after 26 min of a control volume of ethanol (○), 20 μM ethanolic Tinopal AN (●), or 40 μg nalidixic acid ml^{-1} (■).

Fig. 5. The inhibition of RNA synthesis in *E. coli* D22 (*envA1*) by Tinopal AN. RNA synthesis was measured by the uptake and incorporation of [2-^{14}C]uracil by cell suspensions in minimal medium containing 1% disodium succinate. The arrow indicates the addition to the cultures after 15 min of control volumes of ethanol (○) or 20 μM ethanolic Tinopal AN (●).

Effect of Tinopal AN on RNA synthesis in E. coli D22 (envA1)

Synthesis of RNA was measured by the uptake and incorporation of [2-^{14}C]uracil into TCA-insoluble material. Uptake of the label was rapid in exponentially growing cells (Fig. 5). However, addition of 20 μM-Tinopal AN caused an immediate and marked inhibition of uracil uptake and incorporation (and hence of RNA synthesis, see Fig. 5). Furthermore, a rapid decrease in the amount of label already incorporated into the TCA-insoluble material occurred in the presence of Tinopal AN, suggesting that substantial degradation of RNA (the extent implicated ribosomal as well as messenger RNA) occurred.

Effect of Tinopal AN on protein synthesis in E. coli D22 (envA1)

The effect of Tinopal AN on the induction of β-galactosidase production in *E. coli* D22 (*envA1*) was determined using the non-metabolizable inducer IPTG. About 2 min after the addition of inducer, the levels of β-galactosidase increased rapidly (Fig. 6). Addition of control amounts of ethanol caused a slight temporary decrease in the rate of enzyme production. However, addition of 30 μM-Tinopal AN in ethanol caused a rapid, almost immediate, cessation of β-galactosidase synthesis (Fig. 6), suggesting a direct effect of Tinopal AN on a process leading to protein (enzyme) synthesis. The synthesis of protein in exponentially growing cells, measured by the uptake and incorporation of L-[U-^{14}C]proline into TCA-insoluble material, was rapid for the 50 min period of the experiment (Fig. 7). As expected, addition of 20 μM-chloramphenicol, a well-known inhibitor of protein synthesis, caused an immediate inhibition

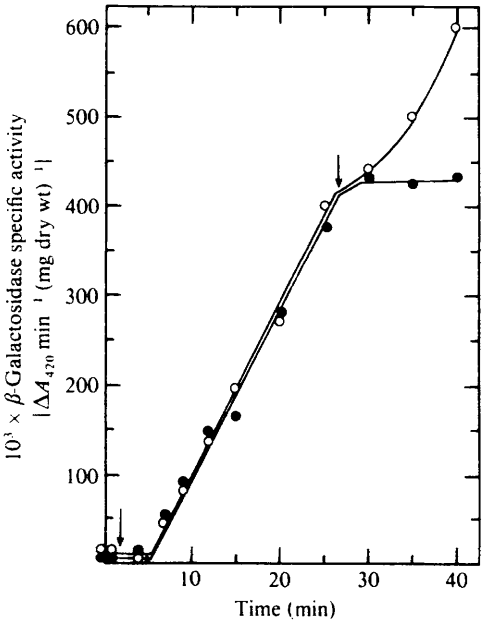

Fig. 6

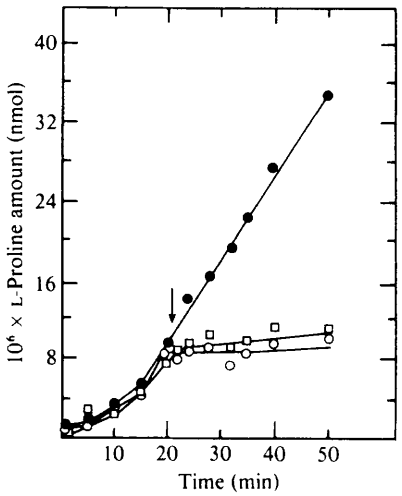

Fig. 7

Fig. 6. The inhibition of the synthesis of β-galactosidase in *E. coli* D22 (*envA1*) by Tinopal AN. The induction of the enzyme by adding the non-metabolizable inducer IPTG after 2 min is shown by an arrow. The arrow at 27 min indicates the addition of a control volume of ethanol (○), or 30 μM-Tinopal AN (●) to the culture. Enzyme activity was measured as described in the text.

Fig. 7. The inhibition of protein synthesis in *E. coli* D22 (*envA1*) by Tinopal AN. Protein synthesis was determined by measuring the uptake of L-[U-^{14}C]proline by exponential phase cultures. The arrow indicates the addition to the cultures of a control volume of ethanol (●), 20 μM-choramphenicol (□), or 20 μM-Tinopal AN (○).

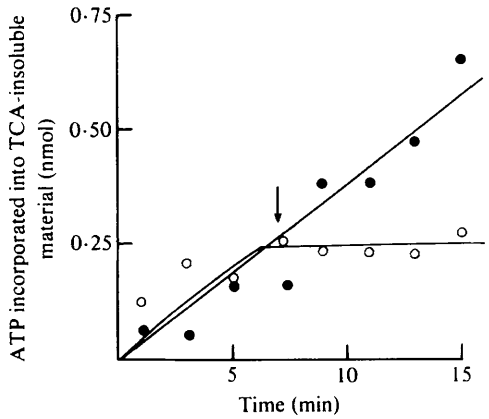

Fig. 8. The effect of Tinopal AN on RNA polymerase activity *in vitro*. RNA polymerase activity was measured as described in Methods. At the time indicated by the arrow, either 0·5% ethanol (●) or 20 μM-Tinopal AN in ethanol (○) was added to the reaction mixture.

of L-proline uptake and incorporation into the cells. Similarly, protein synthesis was rapidly and markedly sensitive to the addition of 20 μM-Tinopal AN (Fig. 7). The results clearly suggest, because the inhibition was immediate, either that Tinopal AN acted by directly inhibiting protein synthesis, or that an immediate inhibition of protein synthesis occurred as an indirect result of the inhibition of RNA synthesis.

Figure 8 shows the effect of Tinopal AN on the incorporation *in vitro* of isotopically labelled

ATP into TCA-insoluble material, catalysed by a purified preparation of RNA polymerase. Tinopal AN acted as a potent inhibitor of this process. The immediate inhibitory effect of this compound both on bacterial growth and on *in vitro* RNA synthesis is consistent with the possibility that Tinopal AN interacted with the ternary DNA–RNA–enzyme complex formed after the initiation of RNA synthesis (see Krakow & Kumar, 1980). However, the RNA degradative activity induced by Tinopal AN in intact *E. coli* D22 (*envA1*) suggests that the compound may have more than one site of inhibitory action *in vivo*. Further studies would be required to determine whether such is indeed the case. Nevertheless, one may conclude from the results presented here that Tinopal AN exerted its bactericidal effect in sensitive bacteria by inhibiting one or more processes leading to protein synthesis.

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