Biochem. J. (1970) **120**, 125–132
Printed in Great Britain

Synthesis of Ribonucleic Acid in Purine-Deficient *Escherichia coli* and a Comparison with the Effects of Amino Acid Starvation

By N. F. VARNEY, GILLIAN A. THOMAS* and K. BURTON
Department of Biochemistry, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K.

(*Received* 26 *May* 1970)

1. Experiments with rifampicin and stringent strains of *Escherichia coli* (*pro*$^-$ *purB*$^-$ *rel*$^+$) indicate that purine deficiency does not decrease and may considerably increase the potential for RNA synthesis by RNA polymerase molecules that are bound to DNA and have already commenced transcription. 2. DNA–RNA hybridization experiments indicate that purine starvation increases the distribution of bound RNA polymerase molecules between the cistrons for mRNA and those for stable RNA. 3. Synthesis of β-galactosidase mRNA is more dependent on the ability to synthesize guanine nucleotides than on the ability to synthesize adenine nucleotides. 4. Amino acid starvation tends to decrease the potential for RNA synthesis by RNA polymerase molecules bound to DNA. 5. Since this effect differs from that due to purine starvation, amino acid control of RNA synthesis does not appear to operate solely by causing a deficiency of purine nucleotides. 6. The results are discussed in terms of the ability to initiate RNA chains and to extend them under different circumstances.

In the preceding paper (Thomas, Varney & Burton, 1970) we showed that the ability to synthesize GTP is more important than the intracellular concentration of ATP in determining the synthesis of RNA during purine deficiency in *Escherichia coli*. In the present paper, we describe experiments with rifampicin to study how far deficiencies of purines or amino acids limit RNA synthesis by affecting the initiation or the growth of RNA chains. We have also studied the synthesis of mRNA by hybridization techniques, the incorporation of amino acids into protein and the synthesis of β-galactosidase. Some of the effects have been compared with those due to amino acid starvation, particularly since Gallant & Harada (1969) have argued that amino acid starvation of stringent bacterial strains (Stent & Brenner, 1961) stops RNA synthesis by limiting the availability of purine nucleotides.

MATERIALS AND METHODS

The bacterial strains and most experimental details are as described in the preceding paper (Thomas *et al.* 1970), bacteria being usually grown aerobically in glucose media at 25°C. For the β-galactosidase experiments, the glucose and NaCl were replaced by 0.1M-sodium lactate to eliminate catabolite repression and the temperature was 37°C. Glycerol was not used as the carbon source since its oxidation can be severely decreased by purine starvation although there is little effect on the oxidation of lactate.

RNA–DNA hybridization analysis for mRNA. After labelling as described in the text, rapidly labelled RNA was isolated for hybridization as described by Pigott & Midgley (1968) except that 10mM-Zn^{2+} was added as a ribonuclease inhibitor before the cell disintegration. DNA was isolated from *E. coli* strain M.R.E. 600 by the method of Pigott & Midgley (1968), who also describe the details of the hybridization technique (Gillespie & Spiegelman, 1965) and ancillary procedures. By following the procedure of Pigott & Midgley (1968) the efficiency of hybridization was found to be approx. 80%. A sample of RNA used for the efficiency determination was included in each series of measurements, as a check on the efficiency. The results are corrected to 100% efficiency as well as for the small amount of non-specific binding.

RESULTS

Effects of purine deficiency on the initiation of RNA chains and on their continued polymerization. Anthony, Zeszotek & Goldthwait (1966), Anthony, Wu & Goldthwait (1969) and Wu & Goldthwait (1969*a*,*b*) have shown that the DNA-dependent RNA polymerase of *E. coli* has two binding sites for purine nucleotide triphosphates; one for initiation and the other for polymerization. *In vitro* the antibiotic rifampicin interacts only with the initiation site (Sippel & Hartmann, 1968; Wu & Goldthwait, 1969*a*,*b*) and this is consistent with the continuation

* Present address: Nuffield Department of Biochemistry, Radcliffe Infirmary, Oxford OX2 6HE, U.K.

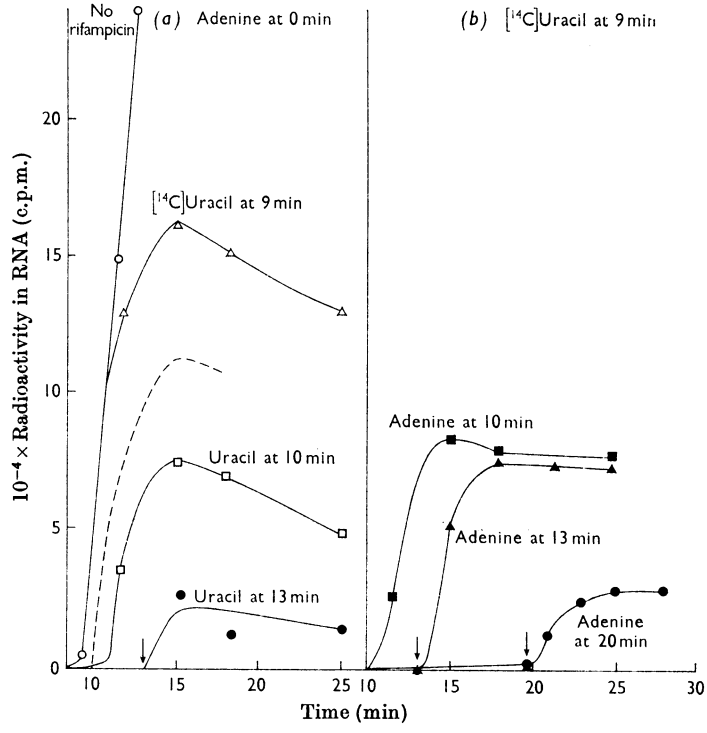


Fig. 1. Incorporation of uracil into RNA and effects of rifampicin after starvation for adenine. At 0 min, washed cells of the *pur B*⁻ strain AB-1325 (1.7×10^{11} cells/l) were suspended in medium lacking adenine. Rifampicin (100 mg/l) was added at 10 min to all portions except one (○). Adenine and [2-^{14}C]uracil (19 μM; 52.5 Ci/mol) were added as follows: (*a*) Adenine at 0 min (○), [^{14}C]uracil at 9 min (△), 10 min (□) or 13 min (●); (*b*) uracil at 9 min and adenine at 10 min (■), 13 min (▲) or 20 min (●). The broken line is the same as curve (△) minus the incorporation at 10 min and thus represents the net incorporation occurring after the addition of rifampicin to a growing culture. The temperature was 30°C.

of RNA synthesis *in vivo* for a few minutes after rifampicin is added to a growing culture (Fig. 1*a*). Assuming that rifampicin instantaneously inhibits the initiation of RNA chains, the continued incorporation represents the potential to synthesize RNA by the completion of chains that had already been started at the time of addition of rifampicin. If rifampicin does not act instantaneously, the potential to complete RNA chains is smaller than is indicated by the peak of the observed incorporation curve.

When rifampicin was added to adenine-deficient cultures, the amount of uracil incorporated into RNA was closely similar whether adenine was restored at the time of addition of rifampicin or after an interval of 3 min (Fig. 1*b*). Hence, until the adenine was added, there was only a small ability to complete RNA chains that had already been started. Moreover, when the adenine and rifampicin were added together, it seems that rifampicin must block the initiation of RNA chains before the adenine stimulates RNA synthesis. The peak of the resulting incorporation curve must therefore represent the potential for completing RNA chains by RNA polymerase that was bound to the DNA when rifampicin was added. Since the peak of the broken line in Fig. 1(*a*) represents the maximum value of this potential in growing cells, it seems that purine starvation can cause only a small decrease in this potential to complete RNA chains.

In an analogous experiment with a *purB*⁻ *guaA*⁻ strain (Fig. 2), purine starvation caused a large increase in the potential for RNA synthesis by DNA-bound RNA polymerase. When either adenine or guanosine was present separately, thus causing an imbalance of purine nucleotides, the potential for RNA synthesis by DNA-bound polymerase was similar to that in exponential growth.

Although rifampicin does not prevent the binding of the enzyme to the DNA (Neuhoff, Schill &

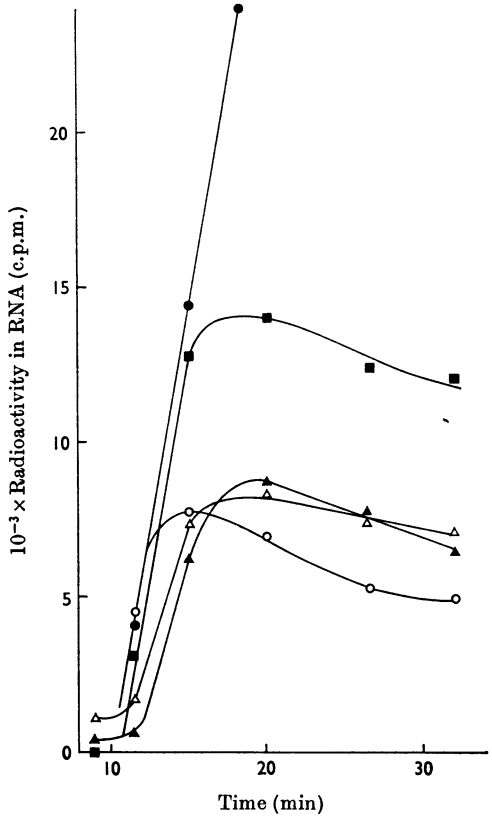


Fig. 2. Effects of rifampicin on the incorporation of uracil into RNA after starvation for adenine and/or guanosine. At 0min, washed cells of the *purB*$^-$ *guaA*$^-$ strain NE-1 (3.2×10^{11} cells/l) were suspended in glucose medium supplemented with histidine and thiamin. Proline was added at 3min. [2-^{14}C]Uracil (19μM; 52.5Ci/mol) was added at 9.5min to two portions (○, ●) that had received adenine and guanosine at 0min and one of which (○) also had rifampicin (100mg/l) added at 10min. All the other portions had [^{14}C]uracil added at 3.5min and rifampicin at 10min, with adenine and guanosine at the following times: ▲, adenine at 0min, guanosine at 11min; △, guanosine at 0min, adenine at 11min; ■, both added at 11min.

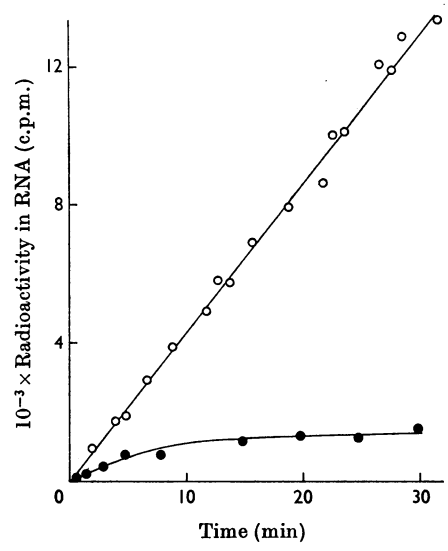


Fig. 3. Labelling of total RNA and of mRNA in exponential-phase cells. Unlabelled uracil (5mg/l) was added to strain AB-1325 (3.2×10^{11} cells/l) growing at 25°C, and, 10min later, [5-^{3}H]uracil (0.5mCi/1; 1.0Ci/mmol) was added: ○, total RNA corrected for the exponential increase in rate; ●, mRNA, also corrected for exponential increase.

Sternbach, 1970), it prevents the subsequent initiation of RNA chains. Thus, the potential to synthesize RNA by DNA-bound RNA polymerase is determined by the number of polymerase molecules that are already transcribing DNA at the time of addition of rifampicin, by the lengths of the segments that are being transcribed and by the distribution of polymerase molecules along them. The most important of these factors is probably the number of polymerase molecules in the process of transcription. It therefore seems that the various forms of purine deficiency limit RNA synthesis at least as much by limiting chain growth as they do by decreasing chain initiation.

Proportions of mRNA and rRNA synthesized. Various methods have been used to study the mRNA content in bacteria (see Midgley, 1969) and, of these, RNA–DNA hybridization seems to be the most reliable (Pigott & Midgley, 1968; Midgley, 1969; Fry & Artman, 1969).

RNA was isolated from cultures of strain AB-1325 at different times after the addition of [5–^{3}H]uracil and hybridized to DNA at a DNA/RNA ratio of 5:1. Controls showed that only about 2% of the rRNA was hybridized under the conditions used and thus, within the precision of the analyses, the hybridized RNA can be taken to represent mRNA.

In agreement with previous workers (e.g. Bolton & McCarthy, 1962; Friesen, 1966, 1968; Stubbs & Hall, 1968; Pigott & Midgley, 1968; Fry & Artman, 1969), mRNA constitutes about 33% of the radioactive RNA made by an exponentially growing culture in the first few minutes of incorporation (Fig. 3). At later times the mRNA is a constant proportion of the total and amounts to 1.7% in strain AB-1325 growing in glucose medium at 25°C. This value is comparable with those obtained by the same method in other strains of *E. coli* (Pigott & Midgley, 1968; Gray & Midgley, 1968), particularly

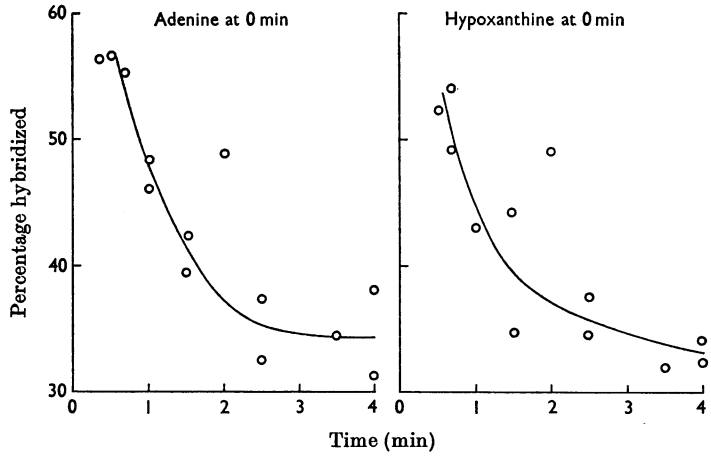


Fig. 4. Proportion of mRNA after addition of purines to a purine-starved culture of strain AB-1325 (*pur B*$^-$). Results are from several experiments in which [5-^{3}H]uracil (4.5–46 μM; 26–280 Ci/mol) was added together with the purine at 0 min. Ordinates show the percentage of the ^{3}H in RNA which was hybridized to DNA after correction for the efficiency of hybridization.

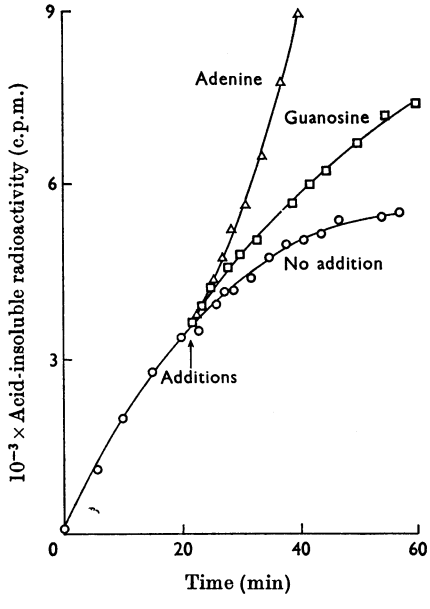


Fig. 5. Incorporation of phenylalanine into acid-insoluble material. Strain AB-1325 was washed and resuspended at 3.4×10^{11} cells/l in purine-free medium at 0 min. L-[U-^{14}C]Phenylalanine (20 μCi/l; 1.64 Ci/mol) was added at 0 min and adenine (△) or guanosine (□) at 21 min (○, no addition). Sample volumes were 0.5 ml.

when allowance is made for a mathematical error as a result of which these earlier values should be decreased by 30%. If the total mRNA is assumed to behave as a homogeneous material and to decay exponentially, the half-life may be calculated to be 5.6 min.

Attempts were made to investigate the nature of the RNA that is labelled in the absence of purines, but insufficient radioactive material was obtained for the hybridization experiment. We therefore studied the RNA that became labelled when [5-^{3}H]uracil was added together with either adenine or hypoxanthine to a purine-starved culture. DNA–RNA hybridization indicated that 60% of the radioactivity in RNA was present as mRNA at the earliest times after stimulation of RNA synthesis by addition of adenine or hypoxanthine (Fig. 4). The experiment also shows that the synthesis of stable RNA (i.e. rRNA and/or tRNA) recovers quickly after the starvation.

Effects on protein synthesis. Although the hybridization experiment indicates that the transient RNA induced by hypoxanthine in the *purB*$^-$ strain includes a high proportion of mRNA, it does not prove that this mRNA is in a functional state. Hence we studied the incorporation of phenylalanine (Figs. 5 and 6) and the synthesis of β-galactosidase (Fig. 7). Both methods showed that protein synthesis in purine-deficient bacteria resembles RNA synthesis in being more dependent on guanine nucleotides than on adenine nucleotides and that it can occur in cells that have a severely decreased ATP content. Since the half-life of the total mRNA is only 5.6 min in strain AB-1325 growing under the conditions used, it is probable that the continued synthesis of protein in purine deficiency is made possible by the continuance of RNA turnover and a

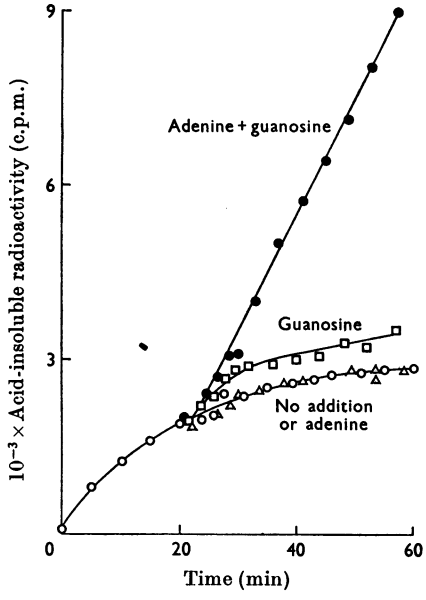


Fig. 6. Incorporation of phenylalanine by strain NE-1. Experimental details are as in Fig. 5 except that cell density was 3.6×10^{11} cells/l: ●, adenine+guanosine; △, adenine; □, guanosine; ○, no addition.

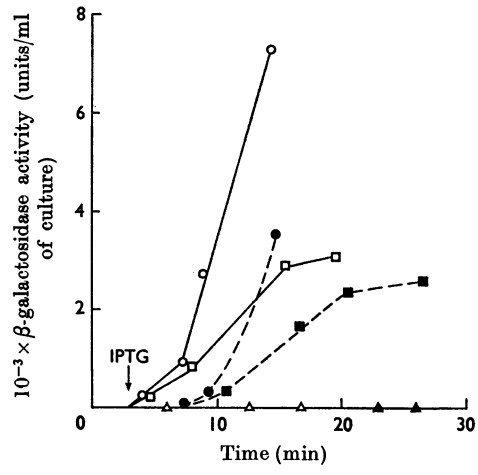


Fig. 7. Induction of β-galactosidase in strain NE-1 (5.4×10^{11} cells/l) at 37°C in lactate medium. Isopropyl β-thiogalactoside (IPTG) (3mM) was added 3min after resuspension of the bacteria in fresh medium and β-galactosidase activity was measured as described by Kepes (1963). Actual β-galactosidase activity is indicated by broken lines and the symbols: ●, ■, ▲. Total β-galactosidase capacity (i.e., activity measured after sampling at the indicated times and incubation for 25min after 50-fold dilution in complete medium): ○, □, △. Complete medium: ○, ●. Medium with guanosine but no adenine: □, ■. Medium with adenine but no guanosine: △, ▲. Zero values were also found with no proline or with both guanosine and adenine omitted.

preferential synthesis of mRNA. Since there is a marked incorporation of amino acids, there must be sufficient GTP for amino acid polymerization.

In the experiment with β-galactosidase, we adopted Kepes' (1963) procedure of measuring both the enzyme activity at different times after addition of inducer and also the total capacity to make enzyme after the inducer had been diluted out and incubation continued for a further 20–25min. This second incubation was in complete medium containing both adenine and guanosine. Hence, the total capacity includes both the amount of completed enzyme and the capacity of the mRNA to support further enzyme synthesis. It would presumably include a contribution from mRNA molecules that have been initiated but not completed at the time of dilution (Kaempfer & Magasanik, 1967). Since there is no capacity to make β-galactosidase in the absence of guanosine, even though amino acids can be incorporated into protein, we conclude that the corresponding mRNA chains are not initiated or else that they are inactivated before they can specify the synthesis of the enzyme.

Comparison of the effects of amino acid deficiency and purine deficiency. Both bacterial strains AB-1325 and NE-1 require proline and they show stringent amino acid control of RNA synthesis. Thus proline starvation decreases RNA synthesis to a low value, although not as low as during adenine starvation (Fig. 8). In our experience proline was required even for the synthesis of β-galactosidase mRNA (Fig. 7) whereas Morris & Kjeldgaard (1968) found that arginine did not at first inhibit the initiation of this RNA in an arginine-requiring stringent strain of *E. coli*.

However, the inhibition of RNA synthesis during amino acid starvation is distinct from that in purine starvation since the potential to incorporate uracil into RNA after addition of rifampicin and restoration of the deficiency is considerably less after amino acid starvation than it is after purine starvation even though the radioactive uracil had been present for several minutes before the addition of rifampicin to the proline-starved culture. In both bacterial strains, the incorporation after the period of proline starvation is less than that observed after addition of rifampicin to a culture in exponential growth (e.g. Fig. 9). The difference between the effects of amino acid starvation and of purine starvation is more marked in the case of strain NE-1 where purine starvation may cause a large increase in the potential for RNA synthesis after the addition of rifampicin (Fig. 2).

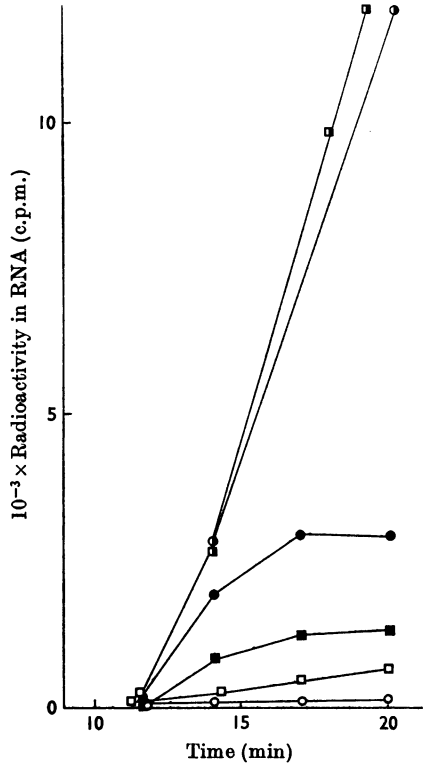


Fig. 8. Comparison of the effects of rifampicin on the incorporation of uracil into RNA after a period of starvation for adenine or proline. Washed cells of strain AB-1325 (2.7×10^{11} cells/l) were suspended in medium lacking proline or purines at 30°C. ○, Adenine-starved, i.e. proline at 0min, [^{14}C]uracil (19μM; 52.5Ci/mol) at 10.5min. □, Proline-starved (adenine at 0min, [^{14}C]uracil at 10.5 min). ◑, Prior adenine starvation (proline at 0min, adenine restored at 10.5min together with [^{14}C]uracil). ●, Prior adenine starvation but rifampicin added at 10min. ◨, Prior proline starvation (adenine at 0min, proline plus [^{14}C]uracil at 10.5min). ■, Prior proline starvation but [^{14}C]uracil added earlier (1min) and rifampicin added at 10min. In this case we have subtracted the small incorporation (2000 c.p.m.) that occurred before rifampicin was added. The curve is virtually identical with that obtained when [^{14}C]uracil was added at 10.5min.

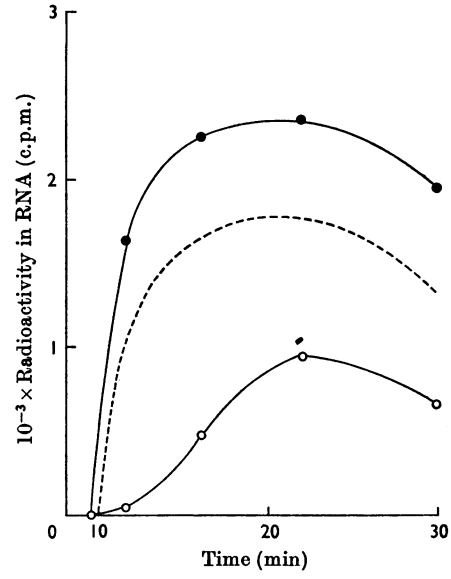
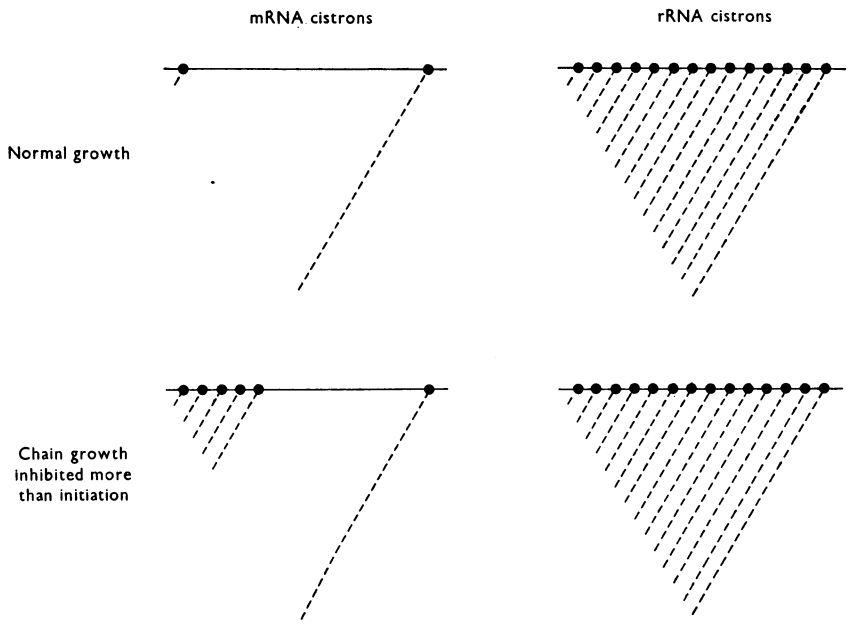


Fig. 9. Effect of prior proline starvation on the incorporation of uracil into RNA in the presence of rifampicin, in washed cells of strain NE-1 (1.2×10^{11}/l) in medium lacking proline. ●, Proline added at 3min, [^{14}C]uracil at 9.5min and rifampicin (100μg/ml) at 10min; ○, [^{14}C]uracil at 8min, rifampicin at 10min and proline at 10.5min. The broken line shows the increase in incorporation after addition of rifampicin to the growing culture.

DISCUSSION

Initiation of RNA chains in purine deficiency. Anthony *et al.* (1966, 1969) and Wu & Goldthwait (1969*a*,*b*) have shown that ATP and GTP bind less readily to the initiation site of *E. coli* RNA polymerase (K_m about 150μM) than to the polymerization site (K_m about 15μM). These properties are apparently not affected by the σ factor (Burgess, Travers, Dunn & Bautz, 1969), since Wu & Goldthwait (1969*a*) obtained the same results after two different purification procedures, one of which should have removed the σ factor.

At first sight, the binding constants might suggest that purine deficiency should limit the initiation of RNA chains more than the polymerization process, particularly since the rate of initiation of the various mRNA chains is probably the principal limiting factor in the total synthesis of mRNA by *E. coli*. However, if RNA synthesis ceases during purine deficiency because of insufficient GTP (Thomas *et al*, 1970), it should still be possible to initiate many RNA chains with pppA- which account for roughly half of the 5′-termini of nascent RNA in *E. coli* (Jorgensen, Buch & Nierlich, 1969). The other 5′-termini are pppG- and we would expect that chains can readily be initiated with these termini during selective adenine deficiency. These arguments are consistent with the observed effects of rifampicin *in vivo* (Figs. 1 and 2) which indicate that purine starvation limits RNA synthesis at least as much by decreasing chain growth as by decreasing the rate of chain initiation. Quite possibly, the *guaA*$^-$ strain, NE-1, may be more severely inhibited in chain growth because it is completely unable to convert adenine nucleotides



Scheme 1. Diagram to illustrate how inhibition of polymerization might increase the number of RNA polymerase molecules bound to mRNA cistrons relative to those bound to rRNA. DNA (the unbroken line) is being transcribed from left to right by RNA polymerase (●). The broken lines represent nascent RNA chains.

into guanine nucleotides, and this may account for the fact that, in strain NE-1, purine starvation has greater effects on amino acid incorporation (Figs. 5 and 6) and also induces a considerable increase in DNA-bound RNA polymerase, as indicated by the rifampicin experiments (Figs. 2 and 9).

Relation between rRNA and mRNA synthesis. After a *purB*$^-$ strain had been starved the proportion of incorporated uracil entering its mRNA was particularly high in the first 30s after addition of either adenine or hypoxanthine. This suggests that adenine starvation increased the number of RNA polymerase molecules bound to cistrons specifying mRNA as compared to the cistrons specifying rRNA. An alternative explanation would involve a separation of the precursor pools for mRNA and rRNA and an easier entry of [^{3}H]uracil into the mRNA pool. However, we know of no evidence for such a separation even though the precursor pools for tRNA and DNA are distinct from those for mRNA and rRNA (Midgley, 1963).

The nature of the initiating nucleotide may be a factor leading to the high proportion of mRNA, but another explanation, shown diagrammatically in Scheme 1, could follow automatically from the general inhibition of ribonucleotide polymerization if the RNA polymerase molecules are normally crowded together on the cistrons for rRNA (Mueller & Bremer, 1968).

Relation between purine deficiency and amino acid control of RNA synthesis. Several workers have examined the effects of amino acid deficiency or of temperature-sensitive aminoacyl transferases on the pool sizes of ribonucleoside triphosphates. Most authors (see Edlin & Broda, 1968; Bagnara & Finch, 1968; Edlin & Stent, 1969) have agreed that any changes are not relevant to the inhibition of RNA synthesis. In the light of our finding that RNA synthesis is very sensitive to the concentration of GTP (Thomas *et al.* 1970) this conclusion may not be justified since several groups have reported marked decreases in GTP. Cashel & Gallant (1968) and Gallant & Harada (1969) have argued very strongly that the stringent amino acid control of RNA synthesis operates by preventing the synthesis of purine nucleoside triphosphates, especially GTP. Against their view is the evidence that large amounts of specific mRNA can be synthesized during stringent control (Edlin, Stent, Baker & Yanofsky, 1968; Lavallé & De Hauwer, 1968) and that lipid synthesis continues (Tropp, Meade & Thomas, 1970).

A marked difference between amino acid and purine starvation was seen when rifampicin plus

proline was added after a period of amino acid starvation. Less uracil was incorporated into RNA than in parallel experiments involving either growing cultures or adenine starvation. This result can be explained most simply by a primary effect of amino acid control on the initiation of RNA chains, but it might also be explained by an inhibition of the incorporation of exogenous uracil that is not completely relieved by restoring the required amino acid in the presence of rifampicin.

G.A.T. thanks the Science Research Council for a Studentship and we are all grateful to the Medical Research Council for their support in the form of the Research Group on the Structure and Biosynthesis of Macromolecules. We thank Lepetit Pharmaceuticals Ltd., Maidenhead, Berks., U.K., for a generous gift of rifampicin.

REFERENCES

Anthony, D. D., Wu, C. W. & Goldthwait, D. A. (1969). *Biochemistry, Easton*, **8**, 246.

Anthony, D. D., Zeszotek, E. & Goldthwait, D. A. (1966). *Proc. natn. Acad. Sci. U.S.A.* **56**, 1026.

Bagnara, A. S. & Finch, L. R. (1968). *Biochem. biophys. Res. Commun.* **33**, 15.

Bolton, E. T. & McCarthy, B. J. (1962). *Proc. natn. Acad. Sci. U.S.A.* **48**, 1390.

Burgess, R. R., Travers, A. A., Dunn, J. J. & Bautz, E. K. F. (1969). *Nature, Lond.*, **221**, 43.

Cashel, M. & Gallant, J. (1968). *J. molec. Biol.* **34**, 317.

Edlin, G. & Broda, P. (1968). *Bact. Rev.* **32**, 206.

Edlin, G. & Stent, G. S. (1969). *Proc. natn. Acad. Sci. U.S.A.* **62**, 475.

Edlin, G., Stent, G. S., Baker, R. F. & Yanofsky, C. (1968). *J. molec. Biol.* **37**, 257.

Friesen, J. D. (1966). *J. molec. Biol.* **20**, 559.

Friesen, J. D. (1968). *J. molec. Biol.* **32**, 183.

Fry, M. & Artman, M. (1969). *Biochem. J.* **115**, 287.

Gallant, J. & Harada, B. (1969). *J. biol. Chem.* **244**, 3125.

Gillespie, D. & Spiegelman, S. (1965). *J. molec. Biol.* **12**, 829.

Gray, W. J. H. & Midgley, J. E. M. (1968). *Biochem. J.* **108**, 34P.

Jorgensen, S. E., Buch, L. B. & Nierlich, D. P. (1969). *Science, N.Y.*, **164**, 1067.

Kaempfer, R. O. R. & Magasanik, B. (1967). *J. molec. Biol.* **27**, 475.

Kepes, A. (1963). *Biochim. biophys. Acta*, **76**, 293.

Lavallé, R. & De Hauwer, G. (1968). *J. molec. Biol.* **37**, 269.

Midgley, J. E. M. (1963). *Biochim. biophys. Acta*, **68**, 354.

Midgley, J. E. M. (1969). *Biochem. J.* **115**, 171.

Morris, D. W. & Kjeldgaard, N. O. (1968). *J. molec. Biol.* **31**, 145.

Mueller, K. & Bremer, H. (1968). *J. molec. Biol.* **38**, 329.

Neuhoff, V., Schill, W. B. & Sternbach, H. (1970). *Biochem. J.* **117**, 623.

Pigott, G. H. & Midgley, J. E. M. (1968). *Biochem. J.* **110**, 251.

Sippel, A. & Hartmann, G. (1968). *Biochim. biophys. Acta*, **157**, 218.

Stent, G. S. & Brenner, S. (1961). *Proc. natn. Acad. Sci. U.S.A.* **47**, 2005.

Stubbs, J. & Hall, B. D. (1968). *J. molec. Biol.* **37**, 289.

Thomas, G. A., Varney, N. F. & Burton, K. (1970). *Biochem. J.* **120**, 117.

Tropp, B. E., Meade, L. C. & Thomas, P. J. (1970). *J. biol. Chem.* **245**, 855.

Wu, C. W. & Goldthwait, D. A. (1969*a*). *Biochemistry, Easton*, **8**, 4450.

Wu, C. W. & Goldthwait, D. A. (1969*b*). *Biochemistry, Easton*, **8**, 4458.