HETEROGENEITY OF RNA POLYMERASE IN *MYCOBACTERIUM TUBERCULOSIS* $H_{37}R_v$

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ABSTRACT

   Crude extracts of M. tuberculosis *cells were fractionated on sucrose gradients of low ionic strengths. In contrast to the sedimentation behaviour of the purified enzyme, the RNA polymerase activity in the crude extracts sedimented heterogeneously over a wide range of the gradient. On raising the ionic strength, the RNA polymerase activity sedimented as two distinct peaks. These two peaks of activity were shown to have different thermal stability, rifampicin sensitivity and affinity for substrates. It is suggested that this physical and functional heterogeneity might have biological significance.*

   Key words : RNA polymerase, Heterogeneity of RNA polymerase, *Mycobacterium tuberculosis.*

INTRODUCTION

   Heterogeneity of bacterial DNA dependent RNA polymerase was first observed by Pene [1] who demonstrated that partially purified *B. subtilis* RNA polymerase could be resolved into two components, a 14 S activity specific for phage DNA and an 18 S activity specific for bacterial DNA. A similar analysis of RNA polymerase in crude extracts of exponentially growing *E. coli* reveals that the enzyme sediments in a broad zone between 51 S and 28 S, with distinct activity peaks at 16–17 S and 21 S [2–4].

   During our attempts to purify RNA polymerase from *M. tuberculosis* [5], we had often observed that the enzyme activity resolved into two peaks on a DEAE-cellulose column. In order to look further into this aspect, we have studied the sedimentation behaviour of the crude enzyme in sucrose gradients. We report here that in crude extracts of *M. tuberculosis* RNA polymerase is heterogeneous and behaves both physically and functionally as though there are two distinct species of the enzyme.

Materials and Methods

Strain.—*M. tuberculosis* $H_{37}R_v$ originally obtained from the National Collection of Type Cultures (NCTC 7416), was used throughout.

Media.—The cells were grown on Sauton's synthetic medium [6], as a surface mat, at 37° C.

Preparation of extracts.—On the eighth day of growth, cells were harvested, suspended in buffer A containing 0·01 M Tris. HCl pH 7·9, 0·001 M $MgCl_2$, 0·01M KCl, 0·1 mM dithiothreitol, 0·1 mM EDTA and 5% (v/v) glycerol (unless otherwise indicated all operations were carried out at 4° C). They were subjected to sonic oscillation in a Raytheon 9 KH_2 sonic oscillator for 20 min. The resulting cell suspension was centrifuged in a Sorvall RC—2B refrigerated centrifuge for 30 min at 12,000 rpm. The supernatant was collected and pancreatic DNase was added to it, to a final concentration of 40 μg/ml and left at 4° C for 2 hr. It was then made 0·5 M with respect to KCl by addition of solid KCl and centrifuged at 100,000 × g for 60 min in a Beckman model L2–50 ultracentrifuge to sediment the ribosomes. The supernatant (S–100) was dialysed overnight against buffer A layered on a 5–15% (w/v) sucrose gradient and centrifuged at 22,000 rpm for 20 hr in a SW 25·1 rotor. After puncturing the tube at the bottom, 1 ml fractions were collected and the RNA polymerase activity was assayed using 0·1 ml fractions. Another portion of the dialysed S–100 fraction was made upto 0·1 M in KCl, layered on a similar gradient in buffer A containing 0·1 M KCl, centrifuged as before and assayed for enzyme activity.

Assay.—RNA polymerase was assayed according to Burgess [7] as described elsewhere [5].

Rifampicin sensitivity.—0·1 ml samples of the enzyme were incubated at 37° C with varying concentrations of rifampicin for 30 min., and assayed for enzyme activity. Rifampicin resistant initiation complexes were assayed as described by Jayaraman [8].

Heat Inactivation.—0·1 ml samples of the enzyme were distributed into several tubes of approximately uniform wall thickness; bovine serum albumin was added to a final concentration of 200 μg/ml, and kept in a waterbath maintained at 50° C. At appropriate times the tubes were removed and chilled in ice. Residual activity was assayed as described above.

RESULTS

Sedimentation of RNA polymerase activity from crude extracts of M. tuberculosis in low and high salt.—Figure 1 shows the sedimentation of RNA polymerase activity fro ma DNase treated, ribosome free extract of *M. tuberculosis* in a 5–15% sucrose gradient containing 0·01 M KCl. The enzyme activity sediments over a broad range which covers nearly two-thirds of the gradient. This suggested that the enzyme was probably heterogeneous, made up of an equilibrium mixture of different aggregates of the e zyme. It is known that the *E. coli* RNA polymerase forms molecular aggregates at low ionic strength and that such aggregates can be broken down to monomers by raising the ionic strength. Therefore the S-100 fraction was recentrifuged on an identical gradient except that the KCl concentration was raised to 0·1 M. Under these conditions the enzyme
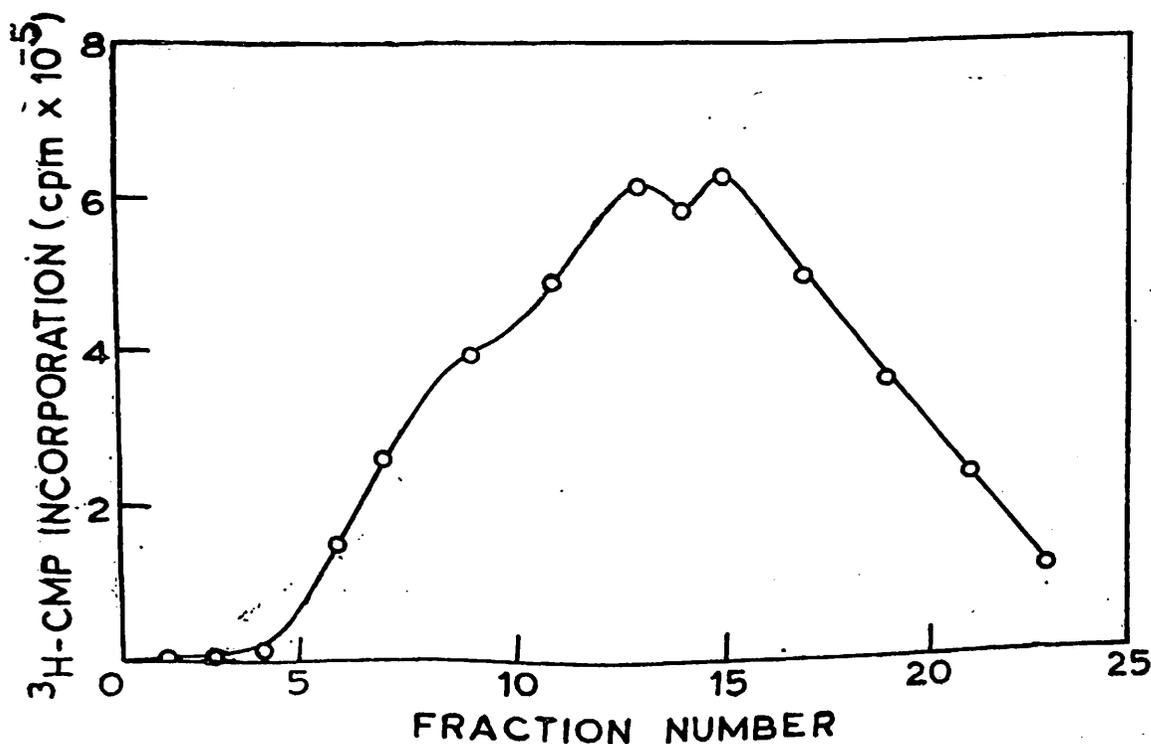

FIG. 1. *Sedimentation of RNA polymerase activity from crude extracts of* M. tuberculosis *in low salt*:

The dialysed S-100 fraction was loaded on a 5–15% sucrose gradient containing 0·01 M KC and centrifuged in a Beckman L2-50 ultracentrifuge at 22,000 rpm for 20 hrs. in a SW$_{25\cdot1}$ rotor. After the run, the tube was punctured at the bottom, 1 ml fractions were collected and assayed for enzyme activity.
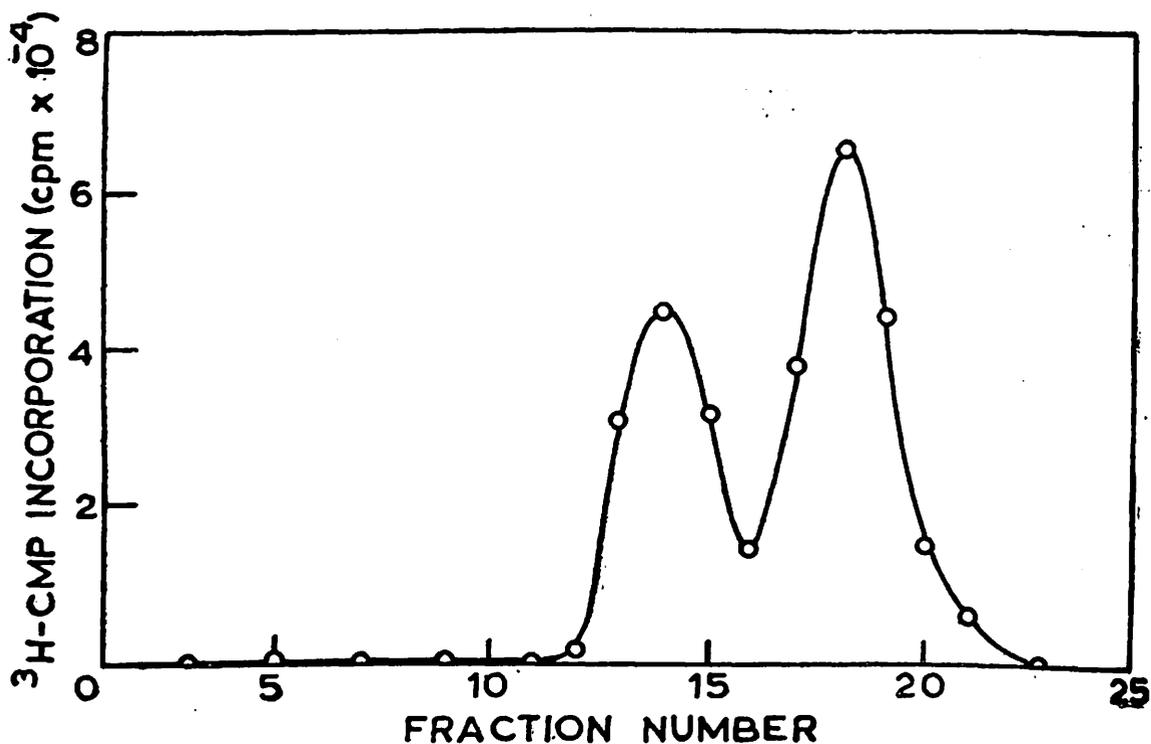
FIG. 2.   *Sedimentation of RNA polymerase activity from crude extracts of* M. tuberculosis *in high salt*:

Details of the experiment are the same as described in the legend for Fig. 1, excepting that the KCl concentration in the gradient was 0·1 M.

activity sedimented as two distinct peaks (Fig. 2). Further increase in KCl concentration in the gradient had no effect on the sedimentation behaviour of these two peaks. The active fractions were pooled and dialysed to remove KCl, and were designated H (fast sedimenting or heavy) and L (slow sedimenting or light) species.

Thermal sensitivity.—The H and L species showed characteristic thermal inactivation profiles which were quite distinct from each other (Fig. 3). These patterns were found to be highly reproducible. The half life of H and L species at 50° were 8 and 21 min respectively. The heat inactivation curves were biphasic for both species. From the plots the half-lives of the fast decaying components of both species were calculated to be approximately same, *i.e.*, 7 min. The slow decaying component of the H species had a half-life of 10 min and that of the L species, of approximately 55 min.
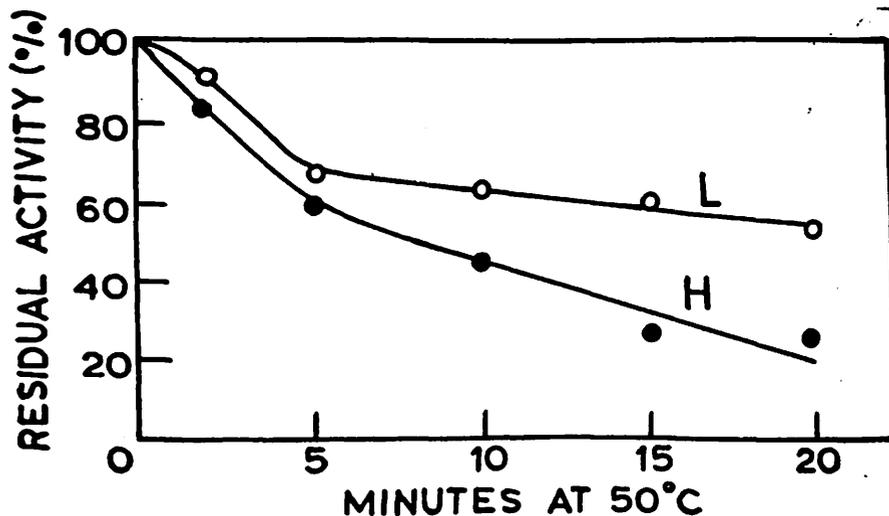
FIG. 3. *Thermal sensitivity of H and L species of RNA polymerase*:
    Details of the experiment are described under 'Methods'. H and L refer to the heavy and light enzyme species.

Sensitivity to Rifampicin.—The H and L species showed differential sensitivity to rifampicin (Fig. 4). The H species was 50% inhibited at $2 \cdot 5 \times 10^{-12}$ M rifampicin whereas the L species required 2×10^{-8} M rifampicin for 50% inhibition.
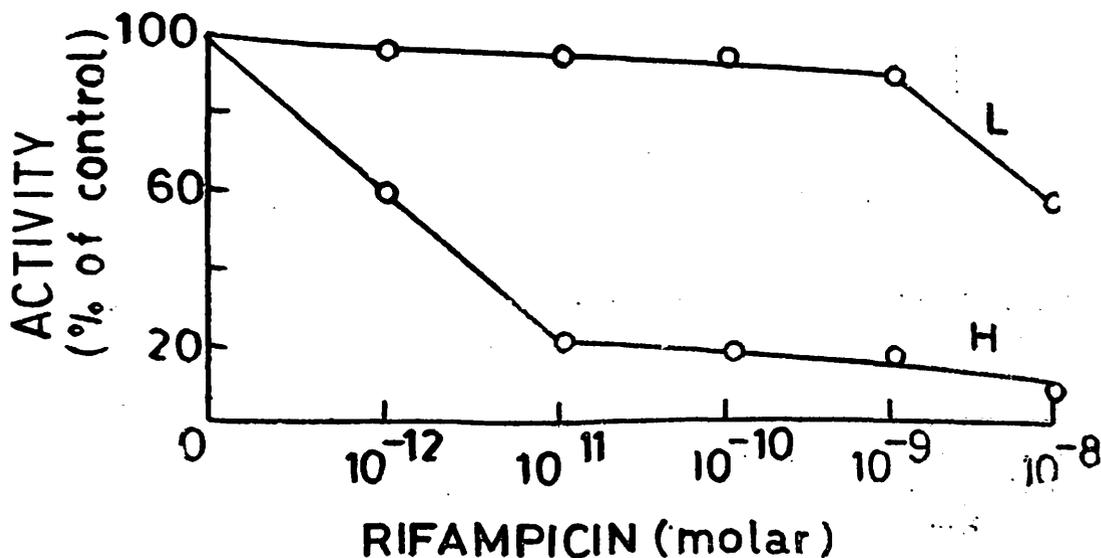

FIG. 4. *Rifampicin sensitivity of H and L species of RNA polymerase*:
Experimental details are given in the text. H—heavy enzyme, L—light enzyme.
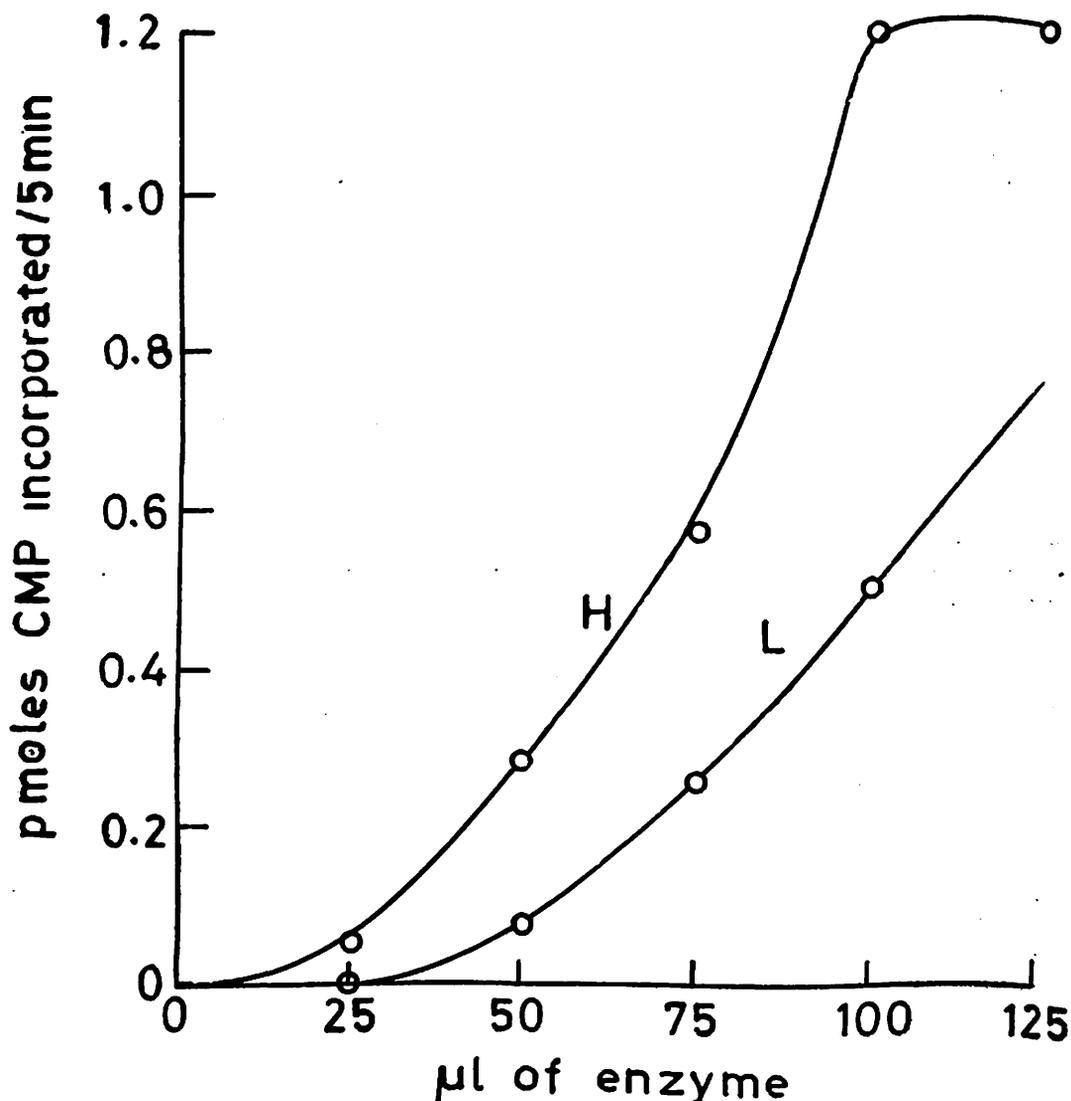
Fig. 5. *Formation of Rifampicin resistant initiation complexes with H and L enzyme species*: The assays were carried out as described earlier [8].

The ability of H and L enzymes to form rifampicin resistant initiation complexes was studied (Fig. 5). Both enzyme species formed these complexes with considerable efficiency, but the kinetics of complex formation were different.

Affinity for UTP and CTP.—The double reciprocal plots of the initial velocity *versus* substrate concentration were constructed for the two enzyme
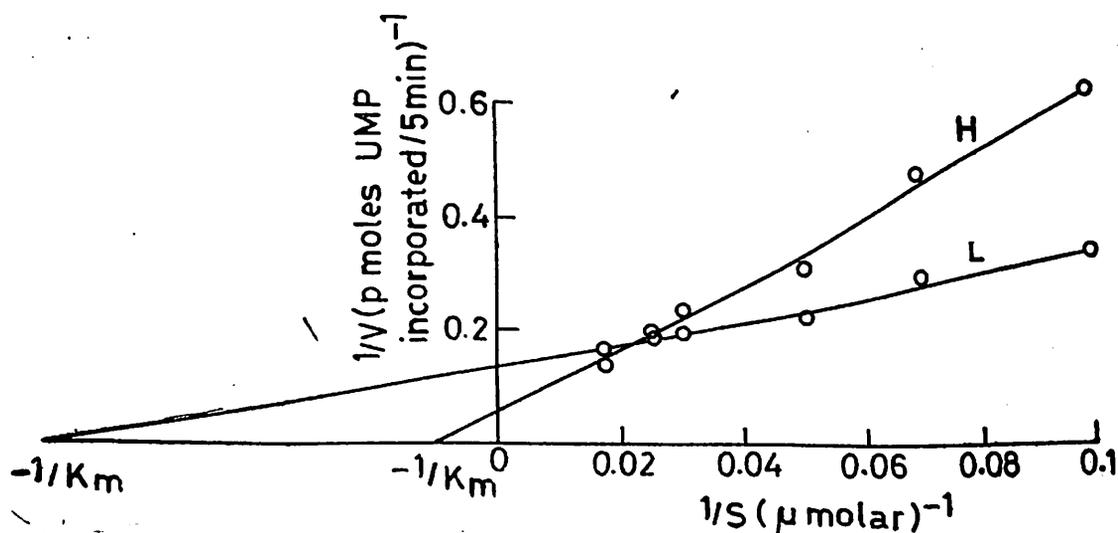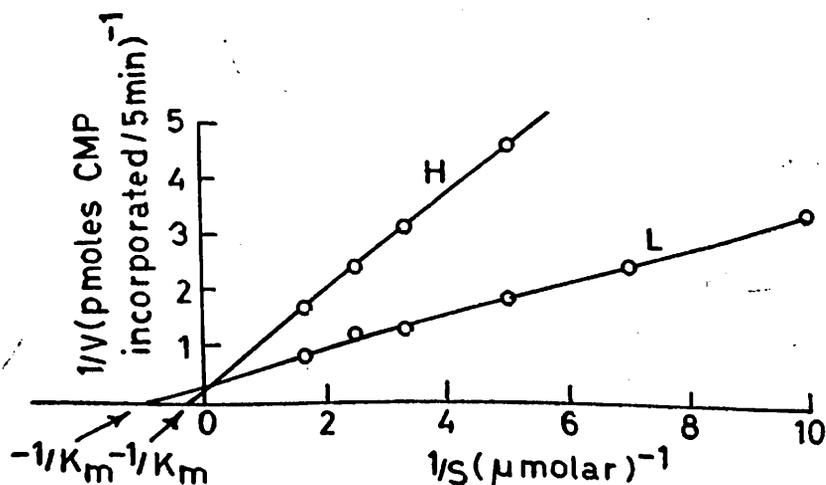
FIG. 6a

FIG. 6b

FIG. 6. *Affinity of H and L species for substrates*:

Enzyme assays were carried out in the usual manner with varying concentration of UTP (*a*) or CTP (*b*) with the other three nucleotide triphosphates held at constant concentrations. The double reciprocal plots were constructed from the substrate concentration *versus* velocity plots.

species (Figs. 6 *a* and 6 *b*). The K_m values for the H and L species for UTP were 1×10^{-4} M and $1 \cdot 5 \times 10^{-5}$ M respectively while those for CTP were 5×10^{-5} M and 1×10^{-6} M respectively.

DISCUSSION

The DNA-dependent RNA polymerase from *Mycobacterium tuberculosis* $H_{37} R_v$ purified by conventional methods bands as a single homogeneous

peak in a glycerol gradient [5]. However, we have presented evidence here to show that in the crude state the enzyme activity sediments heterogeneously in a sucrose gradient. It is likely that this heterogeneity is conferred on the enzyme protein by different factors which are associated with it in the crude state but which are lost during the purification procedure. Such factors have been identified to be present in *E. coli*, e.g., T-Tus [2, 9] and M factor [10], and there are probably as yet unknown ones as well.

The fast sedimenting enzyme fraction (H) is extremely unstable and all our attempts to purify it have proved unsuccessful. The slow sedimenting enzyme (L) is considerably stable and has been successfully purified to homogeneity [5]. The H and L fractions obtained from the sucrose gradient showed differential sensitivity to rifampicin, distinct heat inactivation profiles and differed in their affinities for their substrates UTP and CTP. These results suggest that the physical heterogeneity of the two enzyme species perhaps reflects a functional heterogeneity as well.

The purified RNA polymerase from *E. coli* forms rifampicin resistant initiation complexes when incubated with DNA in the absence of substrates [8, 11–13] and this ability has been considered as a stringent test for the functional integrity of the enzyme [4]. It could also provide a means of testing possible changes in the initiation specificity of the enzyme for different promoters. We have shown here that the kinetics of formation of rifampicin resistant initiation complexes with DNA are quite different for the H and L enzymes. This observation raised the obvious question of whether the two enzymes are involved in the synthesis of specific species of RNA. In *E. coli* it has been shown that the *in vivo* chain growth rate of rRNA is twice as high as that of mRNA [14]. Such an observation is surprising in view of the general assumption that the synthesis of both types of RNA is catalysed by the same enzyme. Our results on rate of RNA synthesis in *M. tuberculosis* [15] indicate that the rate of elongation of stable RNA is twice that of total RNA. However it must be mentioned that the methods employed to measure these two rates were different. Nevertheless the fact that in *E. coli* m RNA is synthesized at a much slower rate than rRNA suggests that the same situation may exist in *M. tuberculosis* also. The first step in the synthesis of RNA is the interaction of the RNA polymerase molecule with a specific promoter site on the DNA. One might expect that alterations in the structure of either the polymerase or the DNA template could affect the rate of synthesis of RNA or the nature of RNA synthesized. Travers and Baillie [16] have shown that the rate of *in vitro*

synthesis of *E. coli* rRNA is dependent on the conformational state of the DNA template. Further, the formation of stable promoter-RNA polymerase complexes is also determined by the nature of RNA polymerase itself [17]. One possible explanation for the intrinsic variability in the initiation specificity of RNA polymerase [15, 18] can be that the enzyme can exist in two conformations, one specific for the initiation of stable RNA synthesis and the other for the initiation of mRNA synthesis. These conformational states can be generated *in vivo* by the interaction of the polymerase with auxiliary specificity factors many of which have already been identified. Among these, ψ now equated with the protein synthesis elongation factor Tu-Ts [9], is thought to stabilize that conformation of RNA polymerase which specifically initiates stable RNA synthesis. In the light of these facts it becomes tempting to speculate that the two species of RNA polymerase that we have observed in *M. tuberculosis* might be of relevance to the synthesis of stable and unstable RNA's in this organism.

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