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A Switch from Translational Control to Transcriptional Control of Protein Synthesis in Mid-exponential Growth Phase of Bacterial Cultures

Specific Radioimmune Labelling of Ribitol-Dehydrogenase-Synthesising Polysomes from *Klebsiella aerogenes* in the Presence of Heparin

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1. We present evidence suggesting a sudden switch from translational control to transcriptional control of protein synthesis in mid-exponential growth of bacterial batch cultures. At a critical cell density a switch from large to small polysomes occurs during a short period of exponential growth. The profile of specific polysomes engaged in synthesis of a constitutive enzyme, ribitol dehydrogenase, changes at the same point but in an opposite way: a linear profile peaking at monosomes changes to a dome-shaped profile peaking at about 15 ribosomes/mRNA, which persists into late exponential phase despite a gradual reduction in the total polysome population. The switch in the pattern of protein synthesis is exhibited dramatically by changes in the specific activity of ribitol dehydrogenase in cell extracts at different stages of batch culture. In early exponential phase the specific activity of the enzyme is constant, but it begins to rise suddenly, at the same point at which the polysome profiles change, and continues to increase up to the end of exponential phase. This effect is exhibited by the strains of *Klebsiella aerogenes* taht are inducible for (in the presence of the inducer), consitutive for, or superproducers of ribitol dehydrogenase, and it appears to be unrelated to catabolite repression.

2. The above results depend on improved techniques for production of large amounts of bacterial polysomes and the ability to label nascent peptides attached to polysomes very specifically with radioactive antibody to ribitol dehydrogenase. Our success was due to the observation that sodium heparin completely abolishes non-specific interactions of the antibody with the polysomes.

It has often been shown that antibodies raised against a pure protein can react with nascent polypeptides of that protein on polysomes [1–7]. This reaction has been used to purify eukaryotic polysomes making a particular protein by a double antibody precipitation technique [8], but the effectiveness of the purification clearly depends on the specificity of the interaction of the antibody with the nascent polypeptide.

Although bacterial messenger RNAs are much less stable than eukaryotic mRNAs, immunoprecipitation of specific polysomes would nevertheless appear to be an attractive route towards purifying quantities of bacterial mRNA from cell extracts. Success depends on a rapid and delicate method of cell lysis that will yield undegraded polysomes, such as the technique of Godson and Sinsheimer [9]. Melcher [10] used this method to isolate polysomes from *Escherichia coli* induced for β-galactosidase, and enriched the β-galactosidase-specific polysomes to 40–50% purity by affinity chromatography on a Sepharose-bound substrate analogue.

Our objective was to identify and purify mRNA specific for the synthesis of ribitol dehydrogenase from extracts of *Klebsiella aerogenes* or *E. coli* K12 strains containing the *K. aerogenes* pentitol operons [11]. This enzyme has been the subject of experimental evolution studies in which extended growth of *K. aerogenes* or *E. coli*/*K. aerogenes* hybrids on xylitol in chemostats focusses selective pressure on a side specificity of ribitol dehydrogenase for xylitol. Two classes of mutants are selected: either those that superproduce wild-type dehydrogenase (up to 30% of the soluble protein in some cases) or strains that make a mutant ribitol dehydrogenase with improved specificity for xylitol [12–15]. We hoped that investigation of ribitol-

Enzymes. Ribitol dehydrogenase (EC 1.1.1.56); lysozyme (EC 3.2.1.17); DNase I (EC 3.1.21.1); lactoperoxidase (EC 1.11.1.7).

dehydrogenase-specific polysomes and mRNA in enzyme-superproducing strains would illuminate the mechanisms of enzyme superproduction.

We have, therefore, used minor modifications of the procedure of Godson and Sinsheimer [9] to isolate large amounts of stable bacterial polysomes from suitable extracts, and have developed methods of specific labelling with purified ^{125}I-anti-ribitol-dehydrogenase to identify polysomes engaged in synthesis of ribitol dehydrogenase is sucrose gradients.

In a strain that produces massive amounts of ribitol dehydrogenase constitutively we observed a sudden change in polysome profiles in mid-exponential phase that coincided with a dramatic shift in the intracellular levels of ribitol dehydrogenase. Our further studies suggest that this is not connected specifically with the phenomena of enzyme superproduction or catabolite repression, but may be a general feature of bacterial batch cultures.

MATERIALS AND METHODS

Reagents

The following reagents were obtained through Sigma Chemicals Ltd: sodium heparin (grade 1), Freund's adjuvants (Difco), chloramphenicol, lysozyme, and sucrose (grade 1: RNase-free). DNase I electrophoretically purified from RNase activity) was purchased from Cambrian Chemicals Ltd (Croydon, Surrey). All radioactive chemicals were purchased from the Radiochemical Centre (Amersham, Bucks).

Bacterial Strains

All *K. aerogenes* strains are derivatives of strain FG5, which is 5P14 of Wu et al. [16]. Strain FG5 is *Klebsiella aerogenes* 1011 (*arg*, *gua*) and is inducible for the ribitol (*rbt*) and D-arabitol (*dal*) catabolic operons. Strain A is *rbt101*, constitutive for ribitol dehydrogenase [12], and is a spontaneous prototrophic revertant of strain XI (*arg*, *gua*, *rbtC101*) of Wu et al. [16]. Strain A111 is a superproducer of ribitol dehydrogenase, derived in three steps from strain A by continuous culture on xylitol [12, 13]. Strain D is *rbt101* and makes a mutant ribitol dehydrogenase, D enzyme, with improved xylitol specificity due to a change of Ala-194 to Pro-194 in its amino acid sequence [13].

Media, Buffers and Culture Conditions

Medium A is 5.8 g Na_2HPO_4, 3.0 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl 0.12 g $MgSO_4$ (added after autoclaving as a 1 M solution), made up to 1 l with distilled water. Medium A is supplemented with sugars or pentitols to 0.2% (w/v) or with Casamino acids to 2% (w/v). Medium B is Bacto tryptone 10 g, Bacto yeast extract 5 g, NaCl 10 g/l. Buffer A is 0.546 g KH_2PO_4, 1.326 g Na_2HPO_4, 7 g NaCl/l, adjusted to pH 7.2 with NaOH.

Culture inoculums were grown overnight (approx. 12 h) with vigorous aeration at 37 °C on the same medium as the final stage. Inoculums of 0.5% (v/v) were used except where otherwise stated.

To avoid contamination with RNase in polysome preparations, all plastic and glassware was soaked thoroughly in detergent and then in dilute HCl, washed with distilled H_2O and autoclaved. Buffers, sucrose solutions, suspensions of ion-exchange resins etc. were made up in sterile H_2O and autoclaved before use. For polysome preparation, sterile plastic ware was used.

Purification of Anti-ribitol-dehydrogenase

Young rabbits were immunised each with 200 µg pure ribitol dehydrogenase D (1 mg/ml) mixed by sonication with an equal volume of Freund's complete adjuvant and injected in multiple subcutaneous and intramuscular sites. Booster injections of 100 µg ribitol dehydrogenase D in Freund's incomplete adjuvant were given every 4–5 weeks. Animals were beld by cardiac puncture 7–14 days subsequent to each booster injection. After allowing the blood to clot overnight, and centrifugation to remove suspended cells, the antisera were stored at −20 °C.

Anti-ribitol-dehydrogenase levels in the antisera were estimated by inhibition of ribitol dehydrogenase activity. Serial dilutions of antisera in buffer A containing 0.5 mg/ml bovine serum albumin were incubated with an equal volume of ribitol dehydrogenase D (10 µg/ml) in the same buffer at 2 °C for 1 h, and samples were then assayed for ribitol dehydrogenase activity [17]. 1 unit of anti-ribitol-dehydrogenase is defined as the amount required to bring about 50% inactivation of 1 µg ribitol dehydrogenase. Antibodies were also quantified by optimal precipitation with pure ribitol dehydrogenase D in double immunodiffusion tests [18].

A ribitol-dehydrogenase – Sepharose affinity resin was prepared by incubating ribitol dehydrogenase D (10 mg/ml Sepharose) with bromoacetamidoethyl-Sepharose-4B for 4 days at 4 °C [19]. After washing with buffer A, about 60% of the protein was found to be coupled, but washing with 1 M acetic acid removed loosely bound enzyme, leaving approx. 2.7 mg strongly bound protein/ml packed Sepharose. This resin (20 ml) was packed into a sterilised column (2 cm diameter) and equilibrated with cold, sterile buffer A.

An immunoglobulin fraction was precipitated from the antisera by adding $(NH_4)_2SO_4$ to 50% saturation and dialysed against buffer A at 4 °C. Samples of this (20–30 mg protein/ml) were applied to an

Table 1. *Purification of anti-ribitol-dehydrogenase by affinity chromatography on ribitol-dehydrogenase-Sepharose*
An Ig fraction [50% satn $(NH_4)_2SO_4$ precipitate from 3 ml rabbit anti-ribitol-dehydrogenase serum was applied to a 0.5-ml bed of ribitol-dehydrogenase–Sepharose-4B, and eluted as shown

Fraction	Total protein	Total anti-ribitol-dehydrogenase	Specific activity
	mg	units	units/mg
Antiserum (3 ml)	210	–	–
Ig fraction applied to column	67.2	8573	127.6
Bound Ig (anti-ribitol-dehydrogenase)	11.4	6000	526
3 M KSCN eluate	2.6	1100	425
1 M acetic acid eluate	1.3	734	569
0.1–1.0 M HCl eluate	0.4	270	730
3 M HCl eluate	0.2	42	191
Precipitate after dialysis	1.7	–	–
Uneluted anti-ribitol-dehydrogenase	5.2	–	–

affinity column of ribitol-dehydrogenase–Sepharose: 3 ml on a test column of 0.5 ml bed volume or 10 ml on a preparative column of 20 ml bed volume. The column was washed with buffer A followed by buffered 0.5 M NaCl to remove weakly bound protein; about 25% of the protein remained strongly bound. Glycine-HCl (0.1 M, pH 2.6) was ineffective in eluting anti-ribitol-dehydrogenase, but a satisfactory elution procedure was 3 M KSCN, followed by 1 M acetic acid, then 0.1 M HCl and finally 1 M HCl, which together eluted more than half of the bound antibody. The KSCN fractions were immediately dialysed versus buffer A, and the acid fractions were neutralised as soon as they eluted and then dialysed versus buffer A.

A typical purification is shown in Table 1 for a 0.5-ml ribitol-dehydrogenase–Sepharose column. About 30% of the eluted anti-ribitol-dehydrogenase precipitates on dialysis, but significant reactivation of the precipitate can be achieved by three cycles of solubilisation in 1 M acetic acid, followed by neutralisation with Tris and dialysis. By these procedures about 50% recovery of the specific anti-ribitol-dehydrogenase could be achieved. It is important to operate such affinity columns near their maximum capacity to obtain the best yield. The column capacity was estimated by titrating a sample of the immunoadsorbent with a standardised anti-ribitol-dehydrogenase serum and measuring the residual anti-ribitol-dehydrogenase activity in the supernatant. About 14000 activity units (26.6 mg) of antibodies to ribitol dehydrogenase bind strongly to 1 ml of the immunoadsorbent; i.e. approximately 2 mol IgG/mol coupled ribitol dehydrogenase monomer. About 50% of the purified antibodies to ribitol dehydrogenase were precipitable at optimal antibody/antigen ratio, and 50 µg ribitol dehydrogenase were optimal for precipitation of 190 µg antibody: a molar ratio of one ribitol dehydrogenase tetramer to about three IgG molecules. Monospecificity of the antibodies for ribitol dehydrogenase was established clearly by double immunodiffusion and immunoelectrophoresis tests (see Results).

^{125}I *Labelling of Antibodies*

Purified anti-ribitol-dehydrogenase (5 mg) in 1 ml cold buffer A, pH 7.8, was treated with (Sigma) lactoperoxidase, 40 µg, in 50 µl buffer A plus 2 µl carrier-free $Na^{125}I$ (100 mCi/ml, 15 mCi/µg ^{125}I) and finally 25 µl 0.1 mM H_2O_2 [20]. Another 25 µl 0.1 mM H_2O_2 was added after 8 min and, after 15 min at room temperature, 1 ml saturated $(NH_4)_2SO_4$ was added to precipitate the antibodies, which were collected by centrifugation and dialysed overnight versus cold buffer A. To remove traces of RNase the antibodies were passed through a small column (0.5 cm diameter) of 1 cm DEAE-cellulose overlaid with 1 cm CM-cellulose. Relative activities of the iodinated and non-iodinated pure anti-ribitol-dehydrogenase were determined by the ribitol dehydrogenase inhibition assay; no apparent loss of activity through iodination was observed. Antibody concentrations were determined using the absorbance $A^{1\%}_{280} = 14.0\,cm^{-1}$ and the radioactivity of ^{125}I-antibody samples was determined in a gamma samples counter.

Preparation of Bacterial Polysomes

For small volumes of cultures the procedure of Godson and Sinsheimer [9] was followed except that chloramphenicol (100 µg/ml) was added 5 s before the cells were harvested. For 1-l cultures of *Escherichia coli* or *K. aerogenes* at any cell density, the following method was used. Chloramphenicol (100 µg/ml) was added to 1 l of a culture at 37 °C. After 5 s the culture was cooled to 0–2 °C in 1 min by pouring it into a chilled 3-l conical flask immersed in methanol/solid CO_2, vigorously swirling the flask to avoid icing and monitoring the fall in temperature. The cooled culture was immediately centrifuged in 4 × 250-ml ice-cooled polycarbonate bottles, in a cold Sorvall GSA rotor at 0 °C for 2 min at 3000 × *g* total acceleration, spinning and deceleration time = 9 min. After discarding the supernatants, the bottles were inverted over Kleenex tissues for 1 min to drain in a freezer, and then the walls were wiped with a tissue to remove residual growth medium. The cell pellets were quickly and thoroughly resuspended in 12 ml 25% (w/v) sucrose in 0.01 M Tris-HCl, pH 8.1, and 3 ml of a freshly mixed solution of lysozyme (0.42 mg/ml) and

EDTA (1.3 mg/ml) in 0.12 M Tris-HCl, pH 8.1, was added. All operations were carried out on ice with precooled solutions in sterile plastic containers or pipettes. The suspension was then added to a lytic mixture prepared 15 min before use containing 3 ml of 5% (w/v) Brij-58 in 0.1 M Tris-HCl, pH 7.2; 3 ml 0.1 M $MgSO_4$; 1.5 ml DNase, 1 mg/ml; 6 ml 1% (w/v) sodium deoxycholate in 0.1 M Tris-HCl, pH 8.1. The cells lyse within a few minutes after mixing, and the lysate was then centrifuged at $6000 \times g$ for 5 min at 0 °C to remove cell debris and membranes.

Samples (15 ml) of the supernatant containing the polyribosomes were layered carefully over a cushion (11 ml) of 1.5 M sucrose in 'polysome buffer' (5 mM Tris-HCl, pH 7.2, 10 mM $MgSO_4$, 60 mM KCl). The tubes were filled with sterile 0.25 M sucrose in 'polysome buffer', and centrifuged at $130000 \times g$ for 12–13 h at 2 °C. After removing the supernatant, the polysome pellet was rinsed gently with 0.25 M sucrose in 'polysome buffer' and suspended carefully in the same medium. After a 5-min centrifugation at $6000 \times g$ the A_{260}/A_{280} ratio of the clear polysome supernatant was measured (ratios of 1.8–1.9 were usually obtained), and it was frozen quickly and stored under liquid nitrogen in sterile plastic tubes. Usually about 15 mg purified polysomes and ribosomes was obtained from a 1-l culture of $A_{650} = 0.6$ (assuming 1 mg polysomes/ml has an $A_{260} = 13$). Such purified bacterial polysomes in sterile solutions are stable for several hours on ice, and for several months when stored under liquid N_2.

Sucrose Density Gradient Analysis of Polysomes

Approximately 0.8 mg purified polysomes was layered carefully, in a minimum volume, onto a cold sterile sucrose gradient (0.5–1.5 M in 56 ml polysome buffer) and ultracentrifugation was carried out in an SW25.2 rotor in a Spinco L2-65B ultracentrifuge at 2 °C for 3 h at 24000 rev./min.

After centrifugation, tubes were pierced at the bottom, and fractions (1.5 ml) were collected at 2 ml/min through a pump and a recording spectrophotometer, filled at first with the same 1.5 M sucrose solution used to make the gradients. Radioactivity of each fraction, in the case of ^{125}I, was measured directly in a gamma radiation counter. For ^{3}H analysis polysomes were first precipitated with trichloroacetic acid, collected on Millipore membrane or glass-fibre filters, washed, dried, and counted in a suitable scintillation fluid on a β-radiation counter.

Specific Activity of Ribitol Dehydrogenase in Cell Extracts

Cells were sampled from growing cultures, pelleted and thoroughly resuspended in 0.1 M Tris-HCl, 5 mM EDTA, pH 8.0 (1 ml/culture $A_{650} = 1.0$). For lower culture densities, bovine serum albumin was added to a final concentration of 1 mg/ml to protect against inactivation of the enzyme. Lysozyme was added to a final concentration of 0.1 mg/ml and, after incubation at 37 °C for 20 min, a solution of 50 mM $MgSO_4$, 2.5% (w/v) Triton X-100, 50 mM Tris-HCl, pH 8.0, was added (12 µl/ml cell suspension). After a further 5 min at 37 °C, the suspension was centrifuged at $17000 \times g$ for 10 min and stored at −20 °C. Ribitol dehydrogenase assays were carried out on samples of these extracts, according to Taylor et al. [21], and protein estimations according to Wang and Smith [22].

RESULTS

a) Characterisation of Purified Antibodies to Ribitol Dehydrogenase

All of the various fractions eluted from the ribitol-dehydrogenase-Sepharose affinity column showed a single precipitin band in immunodiffusion tests [18] against either pure ribitol dehydrogenase D or crude *Klebsiella aerogenes* extracts containing the wild-type enzyme, ribitol dehydrogenase A (data not shown). The crude antiserum also gives a major precipitin band with ribitol dehydrogenase A in such extracts, but at least three other precipitin bands due to impurities in the antigen are present; these are completely removed by the affinity chromatography. Sonicated extracts of cultures of *K. aerogenes* FG5 (inducible for ribitol dehydrogenase), grown without inducer, gave no precipitin bands with purified ribitol dehydrogenase antibody.

Immunoelectrophoresis of the purified anti-ribitol-dehydrogenase versus pure ribitol dehydrogenase D or cell-free extracts containing ribitol dehydrogenase A is shown in Fig. 1. Clearly only one precipitin band corresponding to ribitol dehydrogenase is visible in each dilution of the antibody, either with pure ribitol dehydrogenase or with the sonicated extract. In well 1 (sonicated extract versus 5 mg/ml antibody solution) a small secondary precipitation line is apparent, but this 'spurs' with the main precipitation line, indicating immunological identity with ribitol dehydrogenase. It probably arises from proteolytically 'nicked' ribitol dehydrogenase, which we know is present in significant quantities in such preparations. These experiments clearly show that the purified antibodies are monospecific for ribitol dehydrogenase.

Since the antibody was raised against ribitol dehydrogenase D (which was the purest sample of the enzyme then available to us), it was necessary to demonstrate its reactivity towards ribitol dehydrogenase A, the species synthesised on the polysomes under study. Anti-ribitol-dehydrogenase-D inhibited purified samples of ribitol dehydrogenase A (the wild

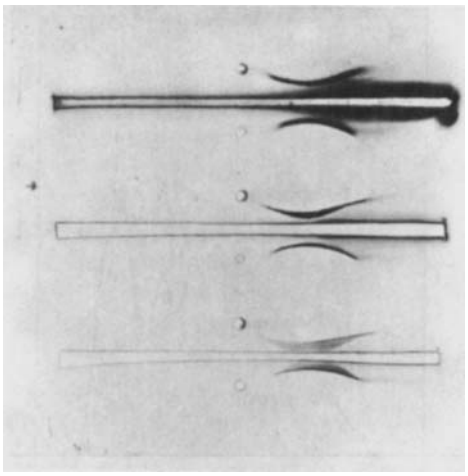

Fig. 1. *Immunoelectrophoresis of purified anti-ribitol-dehydrogenase in 1.5% agar gel (Ouchterlony [18])*. Wells contained alternately, 4-µl samples of sonicated extract of *K. aerogenes* A111 (upper) or 1 mg/ml pure ribitol dehydrogenase (lower). After electrophoresis, a 150-µl sample of purified rabbit anti-ribitol-dehydrogenase was added to each trough; from top to bottom, 5 mg/ml, 1 mg/ml and 0.2 mg/ml respectively

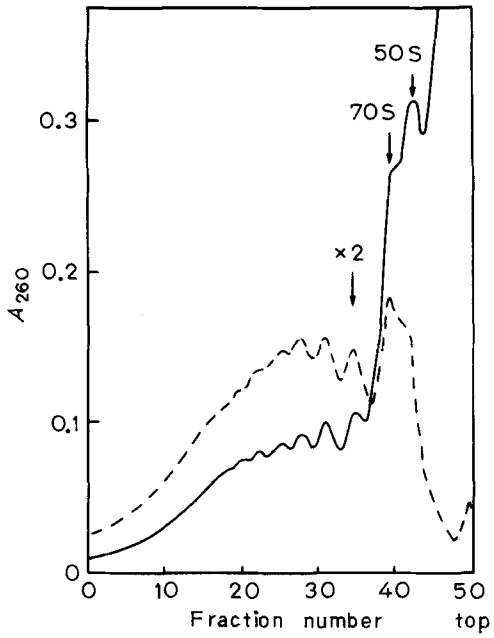

Fig. 2. *Sucrose gradient (15–30%) sedimentation analysis of polysomes from* Klebsiella aerogenes. (——) A_{260} of the 6000 × *g* supernatant of the cell lysate; (– – –) A_{260} of the purified polyribosomes. Ultracentrifugation was conducted in a Beckman L2-65B centrifuge, using an SW 25.2 rotor at 24000 rev./min for 150 min at 2 °C

type) and ribitol dehydrogenase E and ribitol dehydrogenase F (mutant enzymes with increased specificity for xylitol [13]) more strongly than it inhibited ribitol dehydrogenase D. We believe this may be because the former enzyme samples contained inactive protein that reacts less strongly with the antibody, but it certainly indicates that there is strong cross-reaction. Anti-ribitol-dehydrogenase-D inhibited ribitol dehydrogenase EC, the purified enzyme from *Escherichia coli* C [23] with only 37% of its efficiency against ribitol dehydrogenase D. The *E. coli* C enzyme shows approximately 95% sequence homology with the *K. aerogenes* enzyme [23] but it is probable that some antigenic determinants on the surface of the native enzymes will differ.

b) Comparison of Polysomes before and after Purification

In order to show that the purified polysomes are not degraded and have similar size distribution to the polysomes present in a freshly prepared cell extract, bacterial polysomes from a 1-l culture of *K. aerogenes* strain A111 were purified as described in Materials and Methods and stored under liquid nitrogen. Then a 6000 × *g* supernatant from the cell extract of another (80-ml) culture of the same cells grown to the same culture density (A_{650} = 0.55) on the same medium (medium A/xylitol) was prepared. The purified polysomes and the lysate samples were loaded onto parallel sucrose gradients (56 ml each) and ultracentrifuged at the same time.

Fig. 2 shows that the superimposed A_{260} profiles of the ultracentrifuged lysate and the purified polysomes are very similar. The excess A_{260} in the upper fractions of the cell lysate is due to nucleic acids that have diffused into the top fractions of the gradient. The radioactivity and the A_{260} profiles of polysomes prepared from a culture pulse-labelled with [^{3}H]uridine were also very similar to Fig. 2 (results not shown). Hence the purification process does not appear to degrade the polysomes.

c) A Sudden Change in Polysome Profiles at a Critical Culture Density

We have studied extensively the profiles of purified polysomes from cultures at various stages of growth in batch culture, from early exponential to early stationary phase. Profile 1 in Fig. 3 A is typical of polysomes purified from early exponential cultures, up to A_{650} = 0.6 on either B or A/xylitol media, i.e., about 5×10^9 viable cells/ml. Note the broad and rounded polysome dome, peaking at about pentasomes, the shoulder of large polysomes extending to the bottom of the gradient and the relative proportion of the 70-S monosomes to the ribosomal subunits. All the *K. aerogenes* strains that we have investigated produced similar profiles, whether grown on minimal medium (A/xylitol), where the maximum cell density is at A_{650} 3.3, or rich medium (B) where the maximum A_{650} is greater than 20.

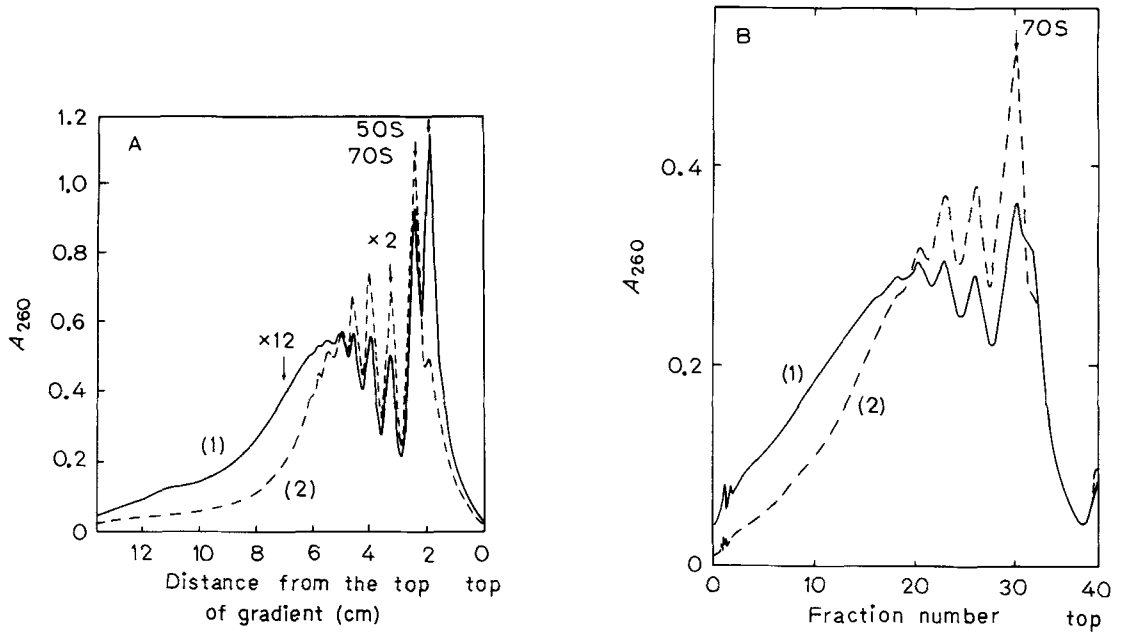

Fig. 3. *Size distributions of purified* K. areogenes *A111 polysomes isolated before or after the 'critical culture density'*. (A) Cells were grown in medium B and harvested for polysome isolation at a culture A_{650} = 0.3 (——) or 0.9 (– – –); polysomes (0.8 mg) were layered onto a 56-ml linear sucrose gradient (0.5–1.5 M) and centrifuged as in Fig. 2, but for 3 h. (B) Cells were grown in medium A/xylitol and harvested for polysome isolation at a culture A_{650} = 0.55 (——) or 0.60 (– – –); polysome sedimentations were conducted in 15–30% sucrose gradients as in Fig. 2

However, the polysomes extracted from the same culture, at an absorbance greater than 0.6, yield a significantly different sedimentation profile, as shown in profiles 2 of Fig. 3A and B. Note that the amount of larger polysomes is greatly decreased, and that there is a significant increase in the proportion of mono, di, tri and tetrasomes but a large reduction in the amount of free ribosomal subunits. The transition from profile (1) to (2) is very rapid: it takes place over a period of 15 min, just before the critical culture absorbance (A_{650} = 0.6) on M9/xylitol as shown in Fig. 3B. On rich medium (B) the change is slightly more gradual, over a range A_{650} = 0.6 to A_{650} = 0.9, but the patterns are essentially similar (compare Fig. 3A and B).

Discrete polysome peaks or inflections are visible in profiles such as Fig. 3A, corresponding to up to 7 ribosomes/mRNA. A double-logarithmic plot of the number of ribosomes versus the distance of the peak from the top of the gradient gives a straight line, which can be extrapolated to give an estimate of the size of larger polysomes (with, of course, progressively decreasing confidence) (Fig. 4). We have used this to estimate size of polysomes in subsequent discussion.

These results suggest a major change in patterns of protein synthesis at a particular point in mid-exponential growth of the culture. Before this the 'early' population of polysomes (> disomes) is very heterogeneous in size; 50% have more than 8 ribosomes attached. Soon after exponential growth is established there is a sudden switch to a 'late' population consisting mainly of oligosomes (1–8 ribosomes) and the proportion of larger polysomes drops by half. There is also a sudden drop in the proportion of ribosomal subunits.

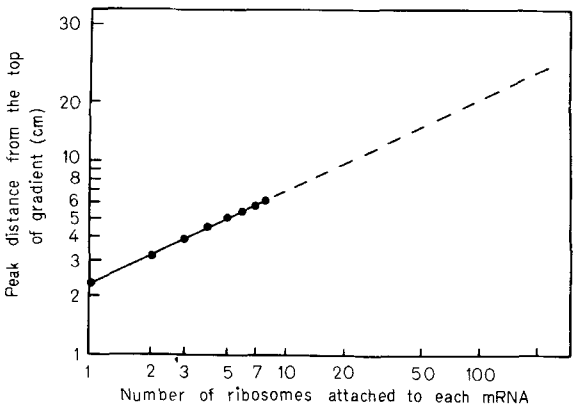

Fig. 4. *Velocity sedimentation distribution of polysomes as a function of size (number of ribosomes/mRNA)*. A double-logarithmic plot of polysome size versus the distance of each observed peak from the top of gradient (data from Fig. 3A)

d) Binding of ^{125}*I-anti-ribitol-dehydrogenase to Purified* K. aerogenes *A111 Polysomes*

Purified bacterial polysome samples were incubated on ice with various amounts of pure ^{125}I-labelled antibodies to ribitol dehydrogenase D. The mixtures were then analysed by velocity sedimentation

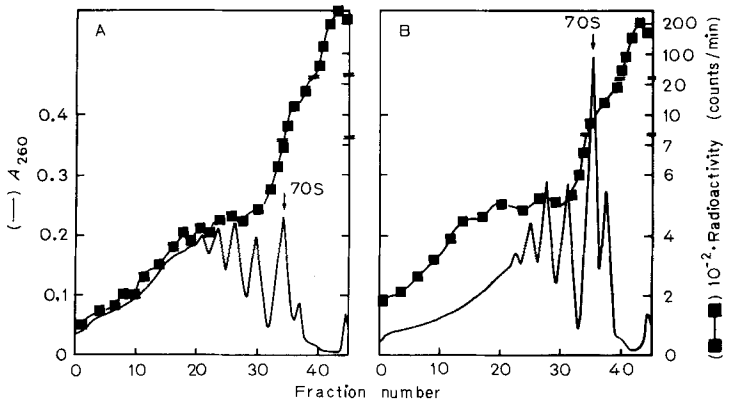

Fig. 5. *Interaction of purified ^{125}I-labelled anti-ribitol-dehydrogenase with purified polysomes from* K. aerogenes *strains FG-5 (A) and A111 (B).* 5 μg iodinated antibodies were incubated on ice with 0.8 mg polysomes in 0.5 ml, for 40 min; then centrifuged as in Fig. 3A for 3.5 h

on linear sucrose gradients. The polysomes were very stable, if appropriately buffered, at all concentrations of ^{125}I-antibodies added (up to 600 μg/mg polysomes tested) and for different incubation periods (up to 1 h investigated), as judged from the comparative sucrose gradient sedimentation profiles with polysomes which were not incubated with the antibodies (profiles not shown). The radioactivity associated with the polysome fractions increase up to about 40 μg antibodies/10 mg polysomes; thereafter the antigenic determinants appear to become saturated with the antibodies.

In order to show that the radioactivity in such polysome profiles truly represents bound antibody rather than artifacts arising from diffusion of antibodies in the density gradient, comparative experiments were carried out with and without polysomes, using equal amounts of the same ^{125}I-antibody solution on identical gradients and by simultaneous centrifugation. There was no diffusion of antibody in the control sample into regions of the gradient greater than 70-S ribosomes.

In an attempt to determine the degree of specificity of interaction of ^{125}I-labelled antibodies to ribitol dehydrogenase with polysomes specifically engaged in ribitol dehydrogenase synthesis we compared purified polysomes prepared from a constitutive ribitol dehydrogenase superproducer strain (A111) or a ribitol-dehydrogenase-inducible strain (FG5). A111 cells were grown on medium A/xylitol medium, while FG5 cells were grown without inducer in medium B.

Both cultures were grown to the same absorbance ($A_{650} = 0.65$). Equal amounts of the two purified polysome samples were incubated with equal quantities of ^{125}I-anti-ribitol-dehydrogenase; then analysed in parallel sedimentation on identical sucrose gradients, as shown in Fig. 5. The general pattern of association of ^{125}I-antibodies with FG5 polysomes (Fig. 5A) is very similar to that with A111 polysomes (Fig. 5B), although the specific activity (counts min^{-1} A_{260} unit^{-1}) of interaction of the iodinated antibodies is double for A111 polysomes than for FG5 polysomes. This suggests that although there is some specific interaction between pure ^{125}I-labelled antibodies to ribitol dehydrogenase and ribitol-dehydrogenase-synthesizing polysomes, there is also an equal amount of non-specific interaction, since the FG5 preparation does not synthesise ribitol dehydrogenase in the absence of inducer.

Since we had shown that our anti-ribitol-dehydrogenase was monospecific, it appeared likely that the non-specific binding was due to general protein–protein or protein–nucleic-acid interactions rather than antigen–antibody interactions. Attempts to eliminate hydrophobic interactions by adding neutral detergents gave little improvement. Schecter [9] achieved high specificity in immunoprecipitation of polysomes, and we noted that he had included heparin in his incubation mixture as a nuclease inhibitor. We reasoned that heparin might also be acting as a competitive inhibitor to prevent salt or magnesium ion bridges responsible for non-specific binding of antibody to polysomes.

The influence of heparin in eliminating the non-specific interactions is shown in Fig. 6. The presence of only 10 units heparin/ml in the antibody/polysome incubation mixtures completely prevented any attachment of purified ^{125}I-anti-ribitol-dehydrogenase to the polysomes from extracts of strain FG5, grown in the absence of an inducer of ribitol dehydrogenase (Fig. 6A). However, under the same conditions the specific binding of the pure anti-ribitol-dehydrogenase to the polysomes carrying nascent ribitol dehydrogenase polypeptides, present in the *K. aerogenes* A111 polysome preparation, becomes clearly evident (Fig. 6B).

Since ribitol dehydrogenase comprises a large proportion of the proteins of *K. aerogenes* A111 cell extract, it may be argued that some of this native

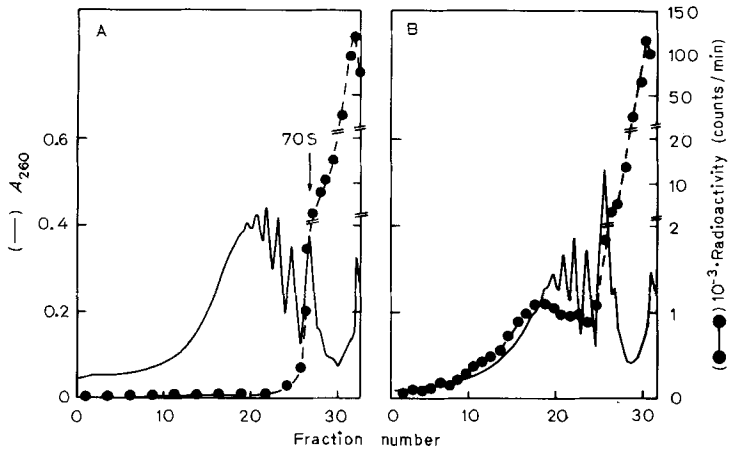

Fig. 6. *Specific interactions of polysomes involved in ribitol dehydrogenase synthesis with ^{125}I-labelled anti-ribitol-dehydrogenase in the presence of heparin.* Polysomes (0.8 mg) in 1.8 ml 'polysome buffer' containing 10 units/ml of sodium heparin and 0.1 M sucrose were incubated for 45 min on ice with 30 µg ^{125}I-anti-ribitol-dehydrogenase (specific activity = 7000 counts min^{-1} μg^{-1}), then centrifuged as in Fig. 3A; (A) purified polysomes from *K. aerogenes* FG-5 strain grown on medium B up to culture A_{650} = 0.6; (B) purified polysomes from *K. aerogenes* A111 strain grown on medium B up to culture A_{650} = 0.75

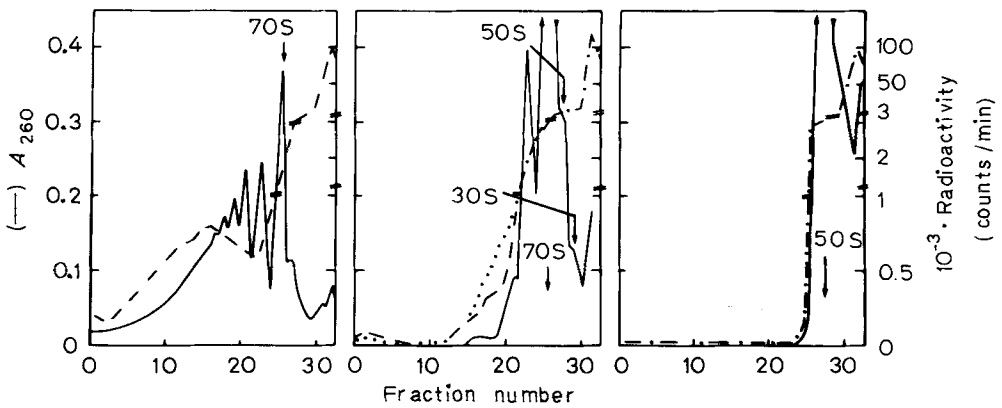

Fig. 7. *Effects of polysome breakdown by RNase and EDTA on the binding of anti-ribitol-dehydrogenase antibodies. K. aerogenes* A111 polysomes (0.3 mg in 0.8 ml) were incubated with 30 µg ^{125}I-labelled anti-ribitol-dehydrogenase antibodies and treated as follows: (A) no disruption; (B) enzymic digestion with 10 µg/ml pancreatic ribonuclease A for 15 min at 2 °C either before (----) or after (·····) antibody incubation; and (C), chemical dissociation with 30 mM EDTA for 20 min either before (----) or after (·····) antibody incubation. Centrifugation conditions are as in Fig. 3A

ribitol dehydrogenase could have contaminated the polysome preparation, and that the observed radioactivity associated with these polysomes on sucrose gradients may be due to sedimentation of antibody/native ribitol dehydrogenase complexes. To prove that this is not the case, tests with RNase and EDTA were conducted. These degrade polysomes, so that antibody bound to polysomes should migrate with the A_{260} profile of the 70-S monosomes, the degraded polysomes, and the ribosomal subunits, whereas radioactivity due to artifactual complexes of ribitol dehydrogenase with antibody would be unaffected. The results presented in Fig. 7, clearly show that the radioimmune-identified material in the sucrose gradients is sensitive to both ribitol dehydrogenase and EDTA.

Furthermore experiments were conducted with polysomes from uninduced strain FG5, mixed with a large excess of native ribitol dehydrogenase (data not shown). When sedimented together with ^{125}I-anti-ribitol-dehydrogenase under the standard conditions, the label appeared either in a precipitate at the bottom of the tube (enzyme-antibody complex?) or remained on top of the gradient (free antibody?); none of the radioactivity appeared in the polysome fraction. Hence there is no non-specific binding of the enzyme to the polysomes.

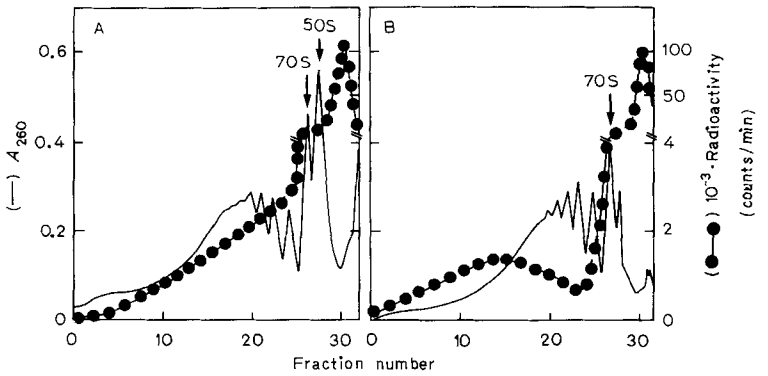

Fig. 8. *Size distribution of ribitol-dehydrogenase-synthesizing polysomes before and after the 'critical culture density'*. A 1-l medium A/xylitol culture of *K. aerogenes* A111 was grown at 37 °C. One half of this culture (A) was harvested at $A_{650} = 0.30$, whilst the other half (B) was harvested at $A_{650} = 0.56$. The cells were lysed and the polysomes purified as described in Materials and Methods. About 0.8 mg of each polysome sample was incubated with excess (30 µg) of ^{125}I-labelled anti-ribitol-dehydrogenase (specific activity about 8000 counts min^{-1} μg^{-1}) in 1.85 ml for 45 min on ice; the sample was then layered carefully onto 56 ml of a linear 0.5–1.5 M sucrose gradient, and the polysomes sedimented as in Fig. 3A

Table 2. *Size distribution of ribitol dehydrogenase polysomes from* K. areogenes *A111 grown on medium A/xylitol, before and after the critical culture* $A_{650} = 0.55$
The calculation of the radioactivity as a proportion of the number of ribosomes in the polysomes was made with the assumption that on the average the same number of antibody molecules attach per nascent peptide from every polysome fraction

No. of ribosomes	At culture $A_{650} = 0.3$			At culture $A_{650} = 0.56$		
	counts/min	counts min^{-1} ribosome^{-1}	%	counts/min	counts min^{-1} ribosome^{-1}	%
1	2300	2300	31.6	0	0	0
2	2300	1150	15.8	100	50	1.8
3	2350	783	10.8	200	67	2.5
4	2200	550	7.6	550	137	5.1
5	2000	400	5.5	700	140	5.2
6	1900	316	4.3	800	134	5.0
7	1800	257	3.5	1000	143	5.3
8	1750	219	3.0	1050	131	4.8
9	1600	178	2.4	1200	134	5.0
10	1450	145	2.0	1250	125	4.6
11–15	1300	100× 5	6.9	1250	96× 5	17.8
16–25	700	35×10	4.6	1200	60×10	22.2
26–35	240	8×10	1.1	1000	33×10	12.2
36–45	0	0	0	700	17×10	6.3
46–55	0	0	0	300	6×10	2.2

e) Sedimentation Profiles of Polysomes Engaged in Ribitol Dehydrogenase Synthesis

We observed above that there appears to be a sudden switch in mid-exponential phase from a heterogeneous population of large polysomes to a persistent population of small oligosomes, peaking at trisomes. We therefore used ^{125}I-anti-ribitol-dehydrogenase as a label to study the distribution of polysomes engaged in ribitol dehydrogenase synthesis at various stages of batch cultures.

Fig. 8A shows a profile typical of cells harvested in early exponential phase, before the 'switch point'. The number of ribitol dehydrogenase polysomes in each fraction decreases linearly as a function of size (number of ribosomes/polysome). Above this cell density the profile of ribitol dehydrogenase polysomes changes suddenly to a dome shape, as shown in Fig. 8 B, coincident with the change in the total polysome profile discussed in section (c) above.

From calibration curves, such as Fig. 4, we can estimate the distribution of ribitol dehydrogenase polysomes in these sedimentation gradients. Table 2 shows that before the 'switch point', about half the ribitol dehydrogenase polysomes are mono- or disomes, and only 13% have more than ten ribosomes attached. After the switch, the proportion of mono and disomes is negligible, and over 60% have more

than ten ribosomes attached. This shift occurs at the same point as the proportion of large polysomes and ribosomal subunits in the total population undergoes a sudden decrease.

A similar change occurs with cells grown on medium B, but here again the change is slightly more gradual, over a range of A_{650} 0.6–0.9. Fig. 9 shows that linear distribution of ribitol dehydrogenase polysomes in early exponential phase shifts to a dome-shaped profile resembling Fig. 8 B. This differs in detail from the profile obtained on medium A/xylitol (Fig. 8 B), since it peaks at 7 ribosomes rather than 15 ribosomes/ribitol dehydrogenase mRNA and extends to only about 25 ribosomes/mRNA against 55 ribosomes/mRNA on medium A/xylitol. Nevertheless the pattern is the same for growth on either rich medium or minimal medium: a switch in mid-exponential phase from large to small polysomes in the total population, which coincides with a switch from small to large ribitol dehydrogenase polysomes. The dome-shaped profile of ribitol dehydrogenase polysomes remains effectively unchanged throughout the remainder of exponential growth, on either rich (medium B) or minimal (medium A/xylitol). For example, Fig. 10 compares the profiles for approximately identical quantities of polysomes harvested during growth on medium A/xylitol just after the 'switch point' (A_{650} = 0.56, or approximately 1 h later (A_{650} = 1.02). Table 3 demonstrates that, although there has been a decay of the total polysome population into monosomes plus ribosomal subunits over this period, the content of ribitol dehydrogenase polysomes remains constant.

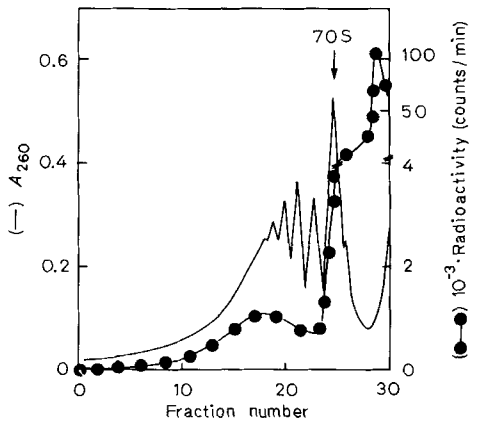

Fig. 9. *Ribitol dehydrogenase polysomes from cells grown on a rich medium.* A 500-ml medium B culture was inoculated by 20 ml of a medium A/xylitol culture of *K. aerogenes* A111 grown to late exponential phase. At a culture A_{650} = 0.9 the cells were harvested and lysed, and the polysomes were purified, incubated with ^{125}I-labelled pure anti-ribitol-dehydrogenase, and sedimented on a sucrose gradient as described in Fig. 3 A

f) Cellular Levels of Ribitol Dehadrogenase at Different Stages of Batch Culture

It was obviously desirable to relate the cellular content of ribitol dehydrogenase to the changes in polysome profiles discussed above. Fig. 11 shows the results for the ancestral ribitol-dehydrogenase-inducible strain (FG5), a ribitol-dehydrogenase-constitutive mutant of this (A) and a number of ribitol dehydrogenase superproducers derived from A (A1, A11, A111 and A112). To facilitate comparisons, these strains were grown on medium A/casamino acids medium, supplemented by inducer (0.2% ribitol) + 0.25 mM guanine for strain FG5. The growth rates of all these strains on this medium are very similar (Fig. 11 C). Samples were withdrawn from the cultures at the absorbances indicated in Fig. 11 A and the

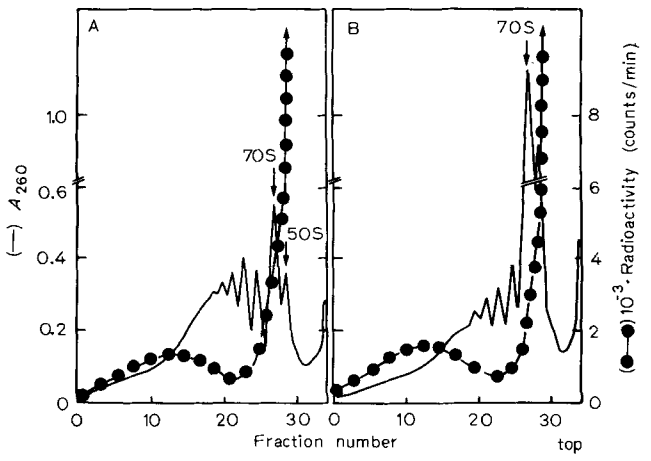

Fig. 10. *Natural decay of the bulk of bacterial polysomes and the stability of the ribitol dehydrogenase polysomes from* K. aerogenes *A111, after the 'critical culture density'.* The cells were grown on medium A/xylitol. The polysome sedimentation profiles, obtained as in Fig. 3 A, correspond to the cells harvested at culture A_{650} = 0.55 (A) and 1.02 (B)

ribitol dehydrogenase activity and total soluble protein was measured in cell-free extracts. The specific activities of ribitol dehydrogenase were very similar whether expressed as units/A_{650} unit of culture (Fig. 11 A) or as units/mg soluble protein (Fig. 11 B) or as units/viable cell (data not shown).

Table 3. *Changes in polysome profiles during exponential growth after the 'switch-point'*
The A_{260} under the curves in Fig. 10 A and B was summed as follows: total ribosomes, fractions 0–32; polysomes, fractions 0–25; oligosomes (disomes to decasomes), fractions 15–25; large polysomes, fractions 0–15. The radioactivity in ribitol dehydrogenase polysomes was summed in fractions 0–22

State of aggregation	A_{260}	
	mid-exponential (culture A_{650} = 0.55)	late-exponential (culture A_{650} = 1.02)
	(%)	(%)
Total ribosomes	12.7 (100)	15.5 (100)
Monosomes + sub-units	4.4 (35)	8.2 (53)
Polysomes	8.3 (65)	7.3 (47)
Oligosomes	6.3 (50)	5.1 (33)
Large polysomes	2.0 (15)	2.2 (12)
Ribitol dehydrogenase polysomes/total ribosomes (counts min^{-1} A_{260}^{-1})	1600	1600

The intracellular level of ribitol dehydrogenase remains essentially constant for all these strains up to the same culture density (A_{650} = 0.7), although the absolute activity is higher for the ribitol-dehydrogenase-superproducing strains. At this critical culture density there is a sudden acceleration in ribitol dehydrogenase/cell, and the increase continues to the end of exponential phase ($A_{650} \approx 4$ on this medium). Similar results were obtained with cells grown on medium A/xylitol medium, where the 'switch-point' appears to be identical to that observed for the change in polysome profiles.

DISCUSSION

We have developed the method of Godson and Sinsheimer [9] to allow preparation of bacterial polysomes on a large scale. The profiles of such polysomes on sucrose density gradients appear to be very reproducible, even after long storage and are identical to the profiles of freshly extracted polysomes prepared on an analytical scale. This reproducibility allowed us to compare profiles of total polysomes at various stages of batch culture of the cells.

We have purified rabbit antibodies that are monospecific for ribitol dehydrogenase by affinity chromatography on ribitol-dehydrogenase-Sepharose. After labelling with ^{125}I, these have proved to be very useful for identifying polysomes engaged in synthesis of this enzyme. However, there was a large amount of non-

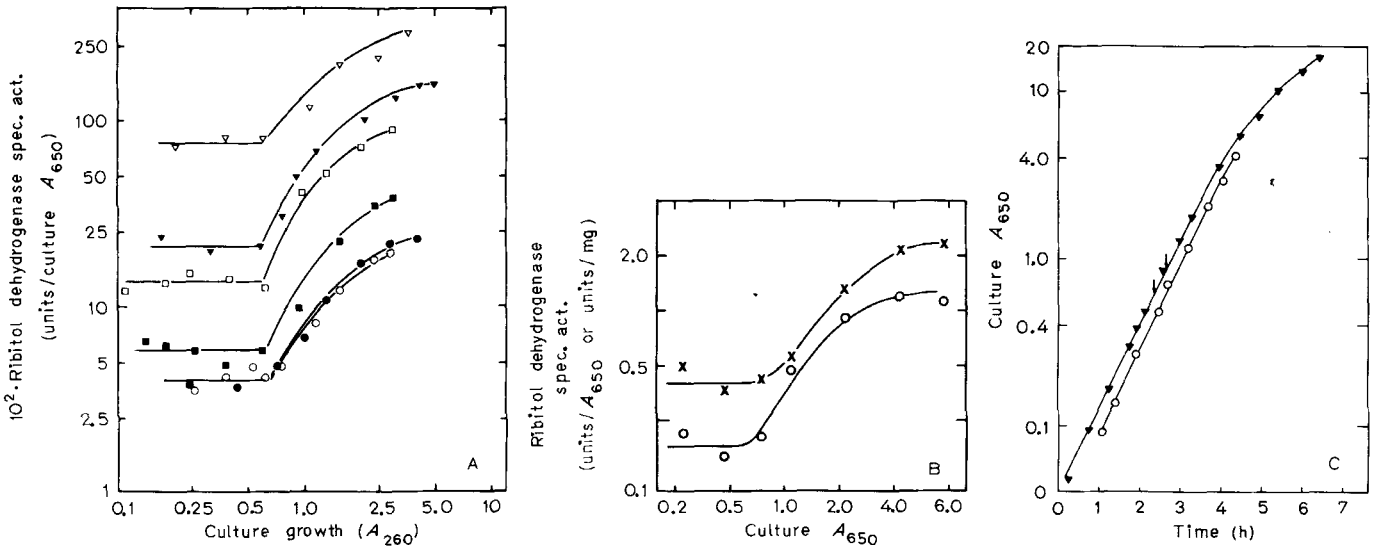

Fig. 11. *Variations in specific activity of ribitol dehydrogenase during growth of various strains of* K. aerogenes. Ribitol dehydrogenase activity [21] was assayed in extracts of cells grown on medium A/casamino acids and harvested at various culture densities. (A) The activity of ribitol dehydrogenase per unit of culture absorbance (A_{650}); (○) the ribitol-dehydrogenase-inducible strain FG5 grown in medium supplemented with 0.2% (w/v) ribitol; (●) the ribitol-dehydrogenase-constitutive strain A. Strain A1 (■), A11 (□), A111 (▼) and A112 (▽) are strains that superproduce ribitol dehydrogenase, selected by continuous culture on xylitol [12, 13]. (B) The specific activity of ribitol dehydrogenase in extracts of strain A111, expressed as either ribitol dehydrogenase units/culture A_{650} (○) or ribitol dehydrogenase/mg soluble protein (×). (C) The growth rates of strain A111 (▼) on medium A/casamino acids, and of strain FG5 (○) on medium A/casamino acids supplemented with 0.2% (w/v) ribitol

specific binding of antibody to the polysomes, that was not eliminated by including detergents in the incubation mixture. These non-specific interactions were completely abolished in the presence of small amounts of heparin. We believe that heparin may be acting as a competitive inhibitor for weak interactions between negative charges on the ribosomes and positive charges on the antibody, or possibly to mask out magnesium ion bridges between antibody and polysomes. Heparin does not appear to interfere with binding of antibody to nascent ribitol dehydrogenase on polysomes, but there was no interaction of the antibody with polysomes from a strain of *Klebsiella aerogenes* inducible for ribitol dehydrogenase synthesis, grown in the absence of the inducer.

Under the conditions described, it is clear that specific labelling of nascent proteins on polysomes can be achieved. By using excess antibodies one nascent peptide will react with one antibody molecule, so that cross-linking of ribosomes by antibodies will be avoided. Hence there will be no tendency to immunoprecipitate the polysomes, and after cleavage of the mRNA by RNase only labelled monosomes will be observed. If unsaturating amounts of antibody are used, divalent antibodies will tend to cross-link adjacent ribosomes on the same polysome string. Hence there will still be no immunoprecipitation by a single antibody so double-antibody precipitation techniques are mandatory [8], but RNase treatment will reveal residual radioactivity in the polysome region. We believe that such criticism may apply to previous experiments attemtping to label nascent proteins on polysomes with antibodies; for example collagen-synthesising polysomes labelled with anti-collagen [24] or fibrinogen-antibody–polysome complexes [25]. In the latter case the authors concluded that the residual radioactivity in the polysome region after RNase treatment indicated assembly of nascent peptides with completed chains before release from the ribosomes. We suggest that such experiments should be repeated with saturating antibody.

We have observed a sudden change in the sedimentation profiles of bacterial polysomes on sucrose gradients at a particular point in early exponential growth in batch culture. The transition occurs in 15 min or less, over a narrow range of culture density: $A_{650} = 0.55-0.60$ on minimal medium (medium A/xylitol) or $A_{650} = 0.60-0.90$ on rich medium (B). At the switch-point there is a big drop in the proportion of large polysomes (8–50 ribosomes/mRNA) and a rise in the proportion of small polysomes (1–8 ribosomes/mRNA), though the total population of ribosomes + polysomes declines by only 20%. The distribution of ribitol dehydrogenase polysomes changes in an opposite sense. Before the switch-point, ribitol dehydrogenase polysomes peak at monosomes and decline linearly to about 35 ribosomes/ribitol dehydrogenase mRNA. The profile changes to a dome-shaped distribution peaking at about 15 ribosomes/mRNA and extending to over 50 ribosomes/mRNA. This distribution of ribitol dehydrogenase polysomes remains constant into late exponential phase, despite a considerable decay in the total polysome population.

These changes in polysome profiles coincide with a dramatic change in the intracellular levels of ribitol dehydrogenase. Before the switch-point the content of ribitol dehydrogenase/cell is effectively constant, though the level is higher in superproducing strains, probably due to gene dosage [12]. After the switch-point the level of ribitol dehydrogenase/cell rises suddenly and continues to rise throughout exponential phase of culture.

These phenomena do not appear to be related to the composition of the medium, since they occur at a similar range of culture densities ($A_{650} = 0.55-0.9$) on minimal (medium A/xylitol) where the maximum $A_{650} = 3.2$, or on rich medium (B) where the maximum $A_{650} > 20$. Nor do they appear to be related to catabolite repression, since similar results are found during growth on minimal/xylitol medium, casamino acids or rich broth. Instead we believe that they reflect an adaptation of stationary-phase cells to steady-state exponential growth, as postulated in the theoretical models of Koch [26]. Koch points out that microorganisms exist in exponential phase growth for but a short fraction of their natural existence. In famine their metabolism is adjusted to minimal survival requirements, hence the level of most metabolic enzymes will be low. However, on introduction to a feast of new substrate adaptation to rapid growth will have selective advantage. Most of the components necessary for rapid protein synthesis seem to be retained in stationary phase, but with *Escherichia coli* there is evidence that levels of some initiation factors are low [27].

When such cells are inoculated into fresh medium, a large pool of new mRNAs will compete for the scarce initiation factors. Hence the pattern of protein synthesis is likely to depend on the concentration of each mRNA and its affinity for the programmed ribosome, i.e. there will be translational control as in the theoretical model of Lodish [28]. However, during this pre-steady state the levels of key metabolic proteins, including of course the initiation factors themselves, will rise gradually until repression begins to limit transcription of the major proteins. At this point there will be less mRNA to compete for the scarce initiation factors, so the cell should move rapidly to a steady-state level of all enzymes. This switch need not coincide with a change in growth rate of the cells, since the protein that limits growth may reach its steady-state level before the bulk of other proteins.

This model could explain the phenomena that we have observed. Before the switch-point a pool of mRNAs would compete for initiation, and a constitutively synthesised (or fully induced) enzyme, such as ribitol dehydrogenase, would have no particular advantage. Hence its level would be more or less in step with cell growth. There would be few ribosomes/mRNA, since initiaton would limit translation.

At the switch-point a jump in the pool of initiation factors would cause a sudden increase in synthesis of the constitutive enzymes and in the size of these particular polysomes. At the same time a shut-down in the rate of transcription of most mRNAs would reduce the size of the average polysome, since there is evidence that oligosomes for small structural proteins predominate in late exponential phase [29].

This hypothesis is clearly not the only one that might explain our observations, but it deserves deeper investigation, and the tools that we have developed for immunolabelling specific polysomes should help.

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