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Abnormal Ribosome Assembly in a Mutant of *Escherichia coli*

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The mutant strain, 15–28, of *Escherichia coli* accumulates ribonucleoprotein ('47S') particles that were previously shown [Markey, Sims & Wild (1976) *Biochem. J.* **158**, 451–456] to be an unusual intermediate in the assembly of 50S ribosomal subunits. In mutant organisms growing at 37°C, pulse-labelling with [^{3}H]uracil and sedimentation analyses show a single precursor to the 47S particles; however, at 20°C, two apparently sequential precursor stages are evident. This contrasts with the synthesis of 50S ribosomal subunits by the parent strain, which, as with other 'normal' strains of *E. coli*, proceeds via two species of precursor that differ in their sedimentation properties from completed subunits and is qualitatively independent of the temperature of growth. Comparison of precursor particles suggests that the pathways of assembly of 50S ribosomal subunits by parent and mutant strains diverge at an early stage.

Escherichia coli strain 15–28 is a mutant whose 70S ribosomes translate endogenous mRNA less efficiently than do ribosomes from its parent, strain 15TP (MacDonald *et al.*, 1967). The peptidyltransferase activity of 50S ribosomal subunits from the mutant is about 70% of that of the parent, and there are differences between parent and mutant organisms in the binding of analogues of peptidyl- and aminoacyl-tRNA (Sims & Wild, 1976; Butler *et al.*, 1978). Concomitant with these functional defects of strain 15–28 is an altered pathway of ribosome assembly. Exponentially growing organisms accumulate unusual ribonucleoprotein particles that lack three of the proteins (L16, L28 and L33) found in completed 50S ribosomal subunits and contain mature (rather than precursor) 23S rRNA (Markey *et al.*, 1976). These properties distinguish the so-called '47S' particles of the mutant from the precursors to 50S ribosomal subunits described by other workers (Blundell & Wild, 1969; Osawa *et al.*, 1969; Nierhaus *et al.*, 1973; Lindahl, 1975). Such precursors invariably contain precursor 23S rRNA and usually lack more proteins than do 47S particles. Nevertheless, kinetic analyses show that the 47S particles are converted into 50S ribosomal subunits during continued growth of the mutant (Markey & Wild, 1976) and so are an unusual intermediate in assembly.

The present paper describes pulse-labelling and sedimentation analyses that compare pathways of ribosome synthesis in parent and mutant strains.

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Assembly of 50S ribosomes by the parent is similar to that described for other wild-type strains of *E. coli* and qualitatively independent of the temperature at which organisms are growing; there are two precursors that differ in their sedimentation properties from 50S ribosomal subunits. However, in the mutant strain, there are striking differences in the assembly of 47S particles (and therefore 50S ribosomal subunits) at different temperatures. At 37°C, there is a single, albeit heterogeneous, precursor that sediments more slowly than 47S particles, whereas in organisms growing at 20°C there are two apparently sequential precursors. It is likely that 47S particles result from an assembly process that, in the mutant strain, diverges at an early stage from the normal pathway of assembly of 50S ribosomal subunits.

Experimental

Growth of bacteria and preparation of cell-free extracts

The organisms were *E. coli thy*$^-$*pro*$^-$ ('15TP') and the mutant, 15–28, derived from it (MacDonald *et al.*, 1967). The bacteria were grown as described previously (Sims & Wild, 1976), in a minimal medium supplemented with casamino acids and with glucose as the carbon source. Experiments used organisms in the mid-exponential phase of growth (A_{450} about 0.4). Cultures (typically 65ml) were first grown for about three generations with 0.5–2.0μCi of [^{32}P]P_i (carrier-free) added per ml of medium (which contains 1.25mM-P_i). At an A_{450} of about 0.4, [^{3}H]uracil (see the Figure legends for details of concentrations) was added to initiate pulse-labelling. Samples (usually

10ml), taken at intervals, were poured on to their own weight of crushed ice at −20°C to stop further incorporation. The cells were harvested by centrifuging at 5000*g* for 15min.

Cell-free extracts were made by a gentle lysis procedure modified from that of Godson (1967) by the omission of Na_2EDTA from the incubation mixtures and by the inclusion of a cycle of freeze–thawing. These modifications are worth comment. When Na_2EDTA was included, lysates from the mutant contained variable quantities of a component that sedimented more slowly than 47S particles and faster than 30S ribosomal subunits. This component is an artefact arising, we think, because sufficient of the chelating agent enters the cells to induce an unfolding to which 47S particles are particularly susceptible (Butler, 1978). Lysis without Na_2EDTA is not as effective as that with the chelating agent present. So the detergent-treated spheroplasts that result from the procedure were routinely frozen in liquid N_2 and then allowed to thaw at 4°C. This increased the effectiveness of the method to that observed when Na_2EDTA was added. The final Mg^{2+} concentration in the lysis mixtures was 10mM.

Gradient centrifuging

The buffer (pH7.4) used in the preparation of sucrose density gradients was 10mM-Tris/HCl/10mM-magnesium acetate/100mM-KCl/1mM-spermidine. Portions (100–250μl) of lysates were layered on 5ml linear or convex exponential sucrose (15–30%, w/w) gradients (Noll, 1967) by using an automatic micropipette with a wide-bore tip. Centrifuging, at 4°C and 234000*g*, used the SW 50.1 rotor of the Spinco model L2-65B ultracentrifuge. Gradients were pumped through an Isco model 185 density-gradient fractionator and collected on to strips of glass-fibre paper (Whatman GF82) as about 50 three-drop fractions. Radioactivity in the fractions was measured as previously described (Markey & Wild, 1976).

Chemicals

Radioactive chemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Deoxyribonuclease I was from Worthington Biochemical Co., Freehold, NJ, U.S.A., Brij 58 was from Honeywill and Stein, Carshalton, Surrey, U.K., and Whatman products were from Whatman, Maidstone, Kent, U.K.

Results

Pulse-labelling at 37°C

For the experiment of Fig. 1, strain 15TP was grown at 37°C with [^{32}P]P_i for three generations and then labelled with [^{3}H]uracil for periods of 25s to 3min 20s. The 'steady-state' profiles that result from the long-term labelling with [^{32}P]P_i are very similar and show tRNA, 'native' 30S and 50S ribosomal subunits and 70S ribosomes; polyribosomes have sedimented. Some ^{3}H radioactivity from the pulse-label is associated with the 70S ribosomes from the earliest labelling time. However, the rate of incorporation of ^{3}H label into the 70S ribosomes increases sharply after about 1.6min (Fig. 1*f*), when newly made [^{3}H]rRNA first appears in this material. This estimate of ribosome maturation time agrees with values of 1.5min (Michaels, 1972) and 1.5–3.5min (Lindahl, 1975) reported for other strains of *E. coli* growing at 37°C.

After labelling with [^{3}H]uracil for 3min 20s (or longer), sufficient synthesis has occurred for the sedimentation profiles for ^{32}P- and ^{3}H-labelling virtually to coincide. However, at the earliest time of labelling (25s) there are present two components that sediment between the 30S and 50S ribosomal subunits and whose sedimentation coefficients are about 37S and 44S. In Fig. 1(*a*), these species are labelled p50S-1 and p50S-2 because of the strong presumption that they are the same as two of the sequential precursors to the 50S ribosomal subunit described by Lindahl (1975), who used this nomenclature. As the labelling time is extended, the ^{3}H radioactivity in p50S-2 particles increases more than that in p50S-1 particles. This is the behaviour expected and described (Mangiarotti *et al.*, 1968; Lindahl, 1975) for these two sequential precursor particles. A single putative precursor to 30S ribosomal subunits ('p30S-1' in Fig. 1*a*; sedimentation coefficient about 27S) is also distinguished by sedimentation, as other work (Schlessinger, 1974) has noted.

At 37°C the mean generation time of strain 15–28 was about 110min, whereas that of its parent (15TP) was about 38min. To accommodate the slower growth of the mutant, pulse-labelling was for longer times. In the experiment of Fig. 2, these ranged from 2.5min to 30min. The ^{32}P-labelled steady-state profiles are those characteristic of the mutant (Markey & Wild, 1976), and show the large quantity of 47S particles relative to 70S ribosomes; as with experiments with the parent strain, the polyribosomes have been sedimented. The rate of increase of the specific radioactivity (^{3}H/^{32}P) of the 70S ribosomes is shown in Fig. 2(*f*). From this, the ribosome maturation time in the mutant is about 24min.

The steady-state profile of the mutant is not its only unusual feature. At the shortest time of pulse-labelling (2.5min), the profile has a rather broad peak of ^{3}H radioactivity between the 30S ribosomal subunits and 47S particles representing a component (labelled 'p47S' in Fig. 2*a*) with an average sedimentation coefficient of about 42S. As the time of labelling is lengthened, radioactivity in this region increases; so does the average sedimentation coefficient, until the sedimentation of the ^{3}H pulse-label virtually

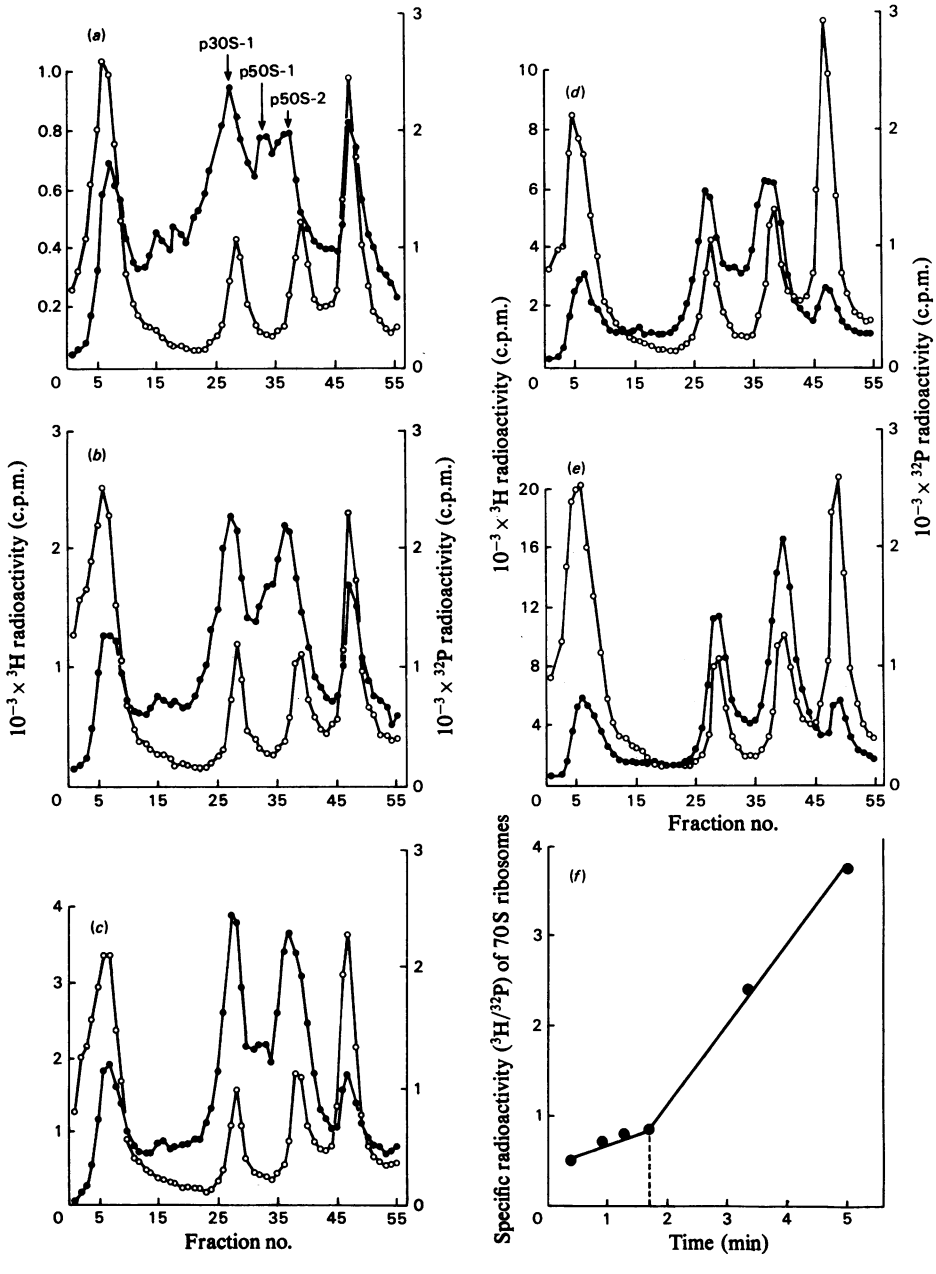

Fig. 1. *Pulse-labelling of strain 15TP at 37°C*

A culture (65ml) of strain 15TP was grown to A_{450} 0.1, and then [^{32}P]P_i (2μCi/ml) was added. At A_{450} 0.35, the culture was divided into six 10ml portions. Growth of these continued to A_{450} 0.4, when each received [^{3}H]uracil [25μCi (1μg)/ml] for the times indicated. The growth of a culture was stopped by pouring on to crushed ice at −20°C. The cells from each sample were collected by centrifugation, and lysed (final volume 0.5ml). A portion (200μl) of each lysate was centrifuged for 165min at 234000*g* through a 5ml linear sucrose gradient. Times of ^{3}H-labelling were (*a*) 25s, (*b*) 50s, (*c*) 1min 15s, (*d*) 1min 40s, (*e*) 3min 20s. ○, ^{32}P radioactivity; ●, ^{3}H radioactivity. Part (*f*) shows the specific radioactivity (^{3}H c.p.m./^{32}P c.p.m.) of the 70S ribosomes in panels (*a*)–(*e*) plotted against the time of exposure to [^{3}H]uracil.

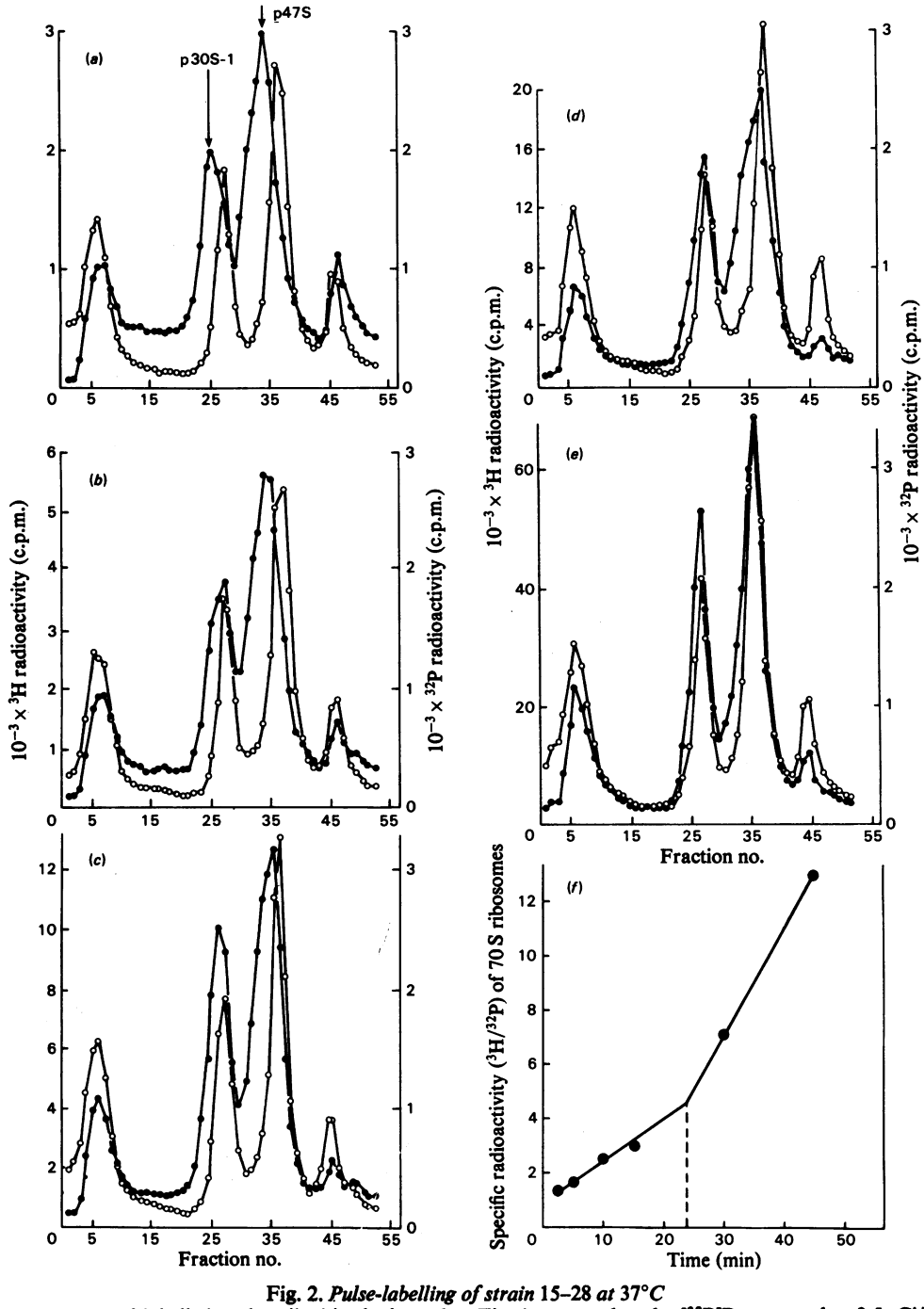

Fig. 2. *Pulse-labelling of strain* 15–28 *at* 37°C

Cells were grown and labelled as described in the legend to Fig. 1, except that the [^{32}P]P_i was used at 0.5 μCi/ml and the [^{3}H]uracil at 12 μCi (5 μg)/ml. Portions (150 μl) of the lysed cells were centrifuged for 165 min at 234000*g* through 5 ml linear sucrose gradients. Times of ^{3}H labelling were (*a*) 2.5 min, (*b*) 5 min, (*c*) 10 min, (*d*) 15 min, (*e*) 45 min. ○, ^{32}P radioactivity; ●, ^{3}H radioactivity. Part (*f*) shows the specific radioactivity of the 70 S ribosomes in panels (*a*)–(*e*) plotted against time of exposure to [^{3}H]uracil.

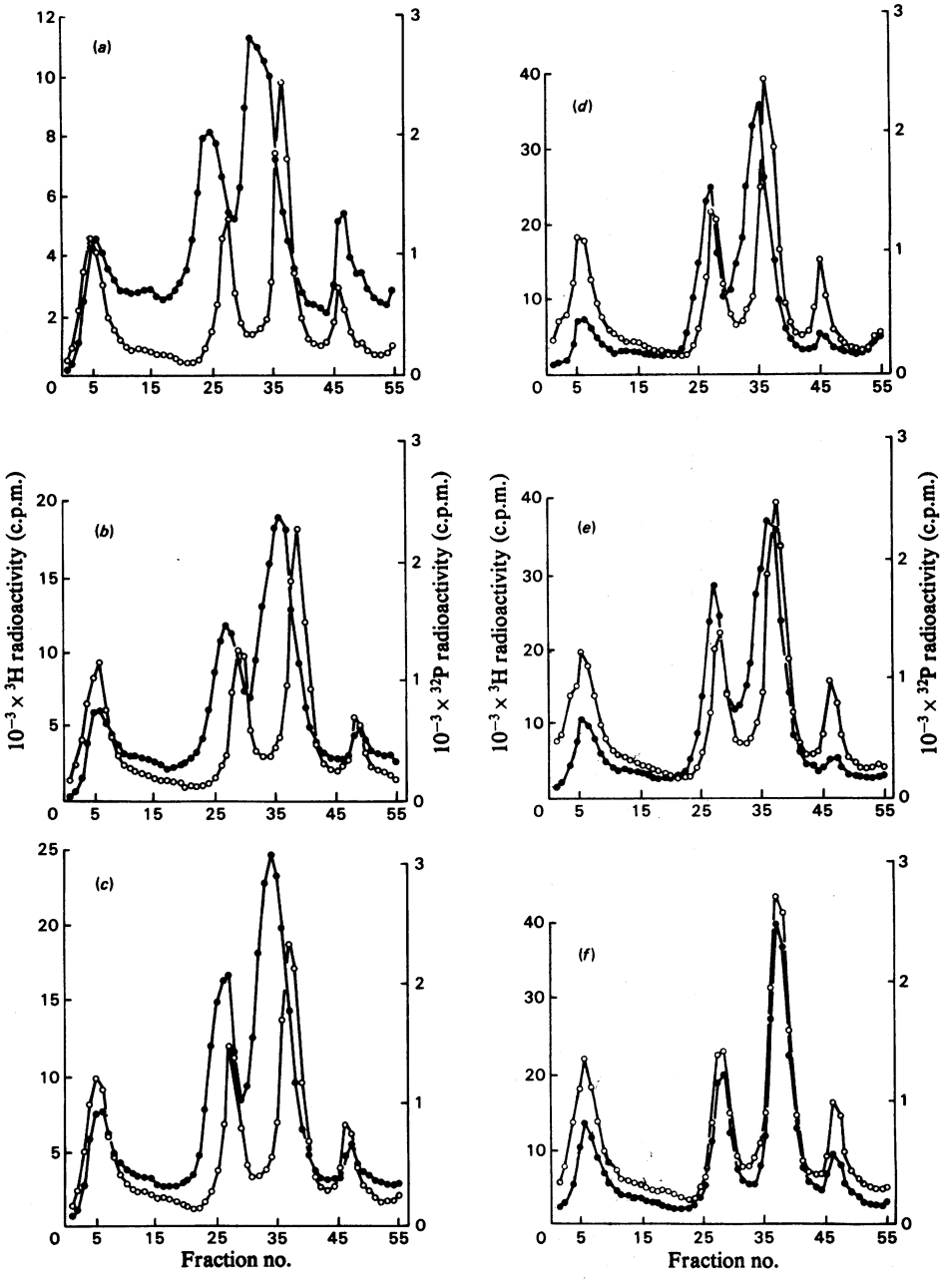

Fig. 3. *'Pulse–chase' experiment with strain* 15–28

A culture (65ml) of strain 15–28 was grown at 37°C for two generations to A_{450} 0.4 with [^{32}P]P_i (0.5μCi/ml) present. Carrier-free [^{3}H]uracil (26Ci/mmol) was added to give 5μCi (22ng)/ml. After 1min, non-radioactive uracil (final concn. 100μg/ml) was added. A sample of the culture (10ml) was taken immediately and poured on to crushed ice at −20°C. Further samples were taken at the times below. The cells from each sample were collected by centrifuging and lysed (final volume 0.5ml). Samples (100μl) of each lysate were centrifuged for 165min at 234000*g* through 5ml linear sucrose gradients. Times of sampling (measured from addition of non-radioactive uracil) were (*a*) 0min, (*b*) 1min, (*c*) 2min, (*d*) 5min, (*e*) 10min, (*f*) 45min. ○, ^{32}P radioactivity; ●, ^{3}H radioactivity.

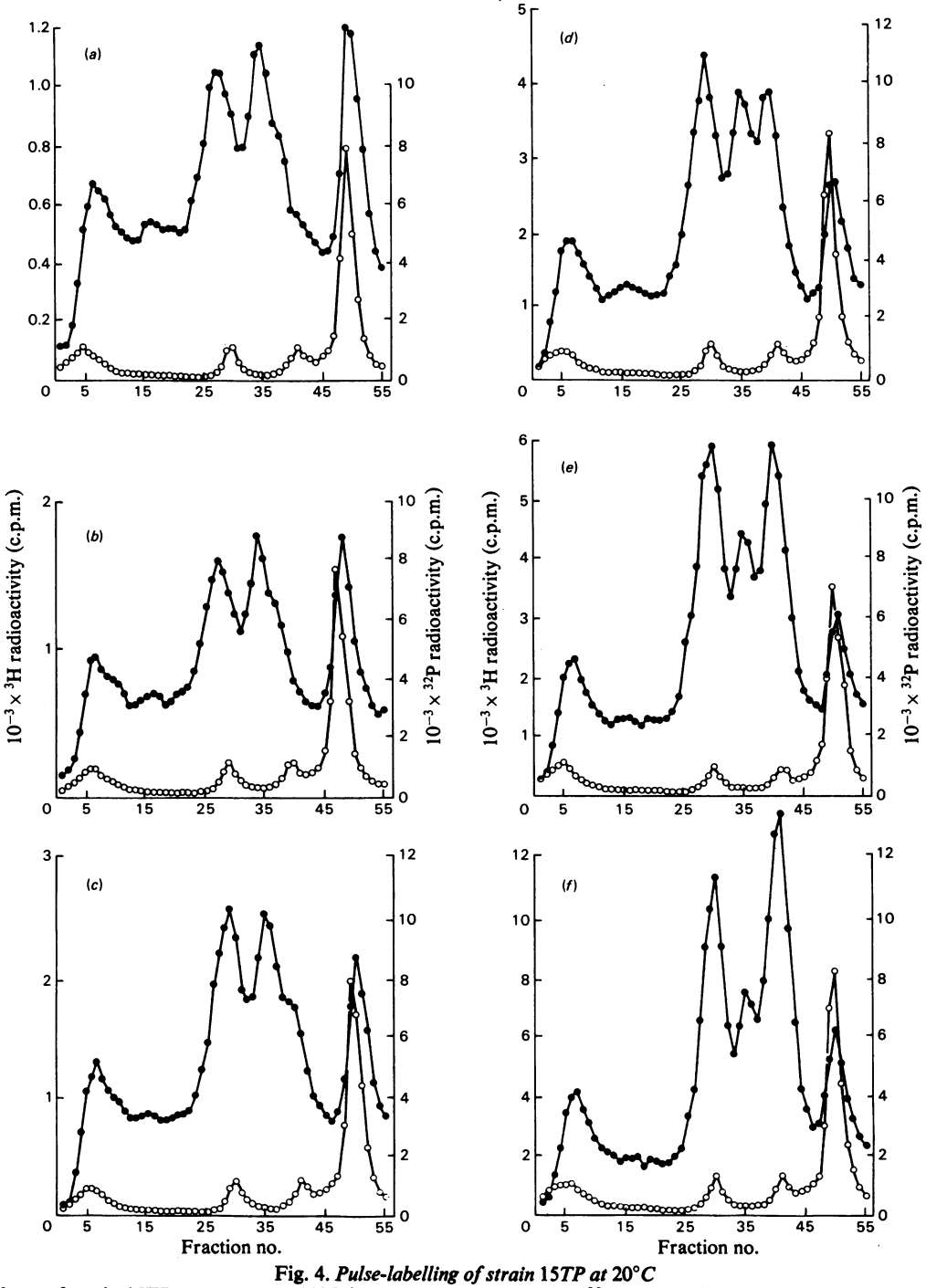

Fig. 4. *Pulse-labelling of strain 15TP at 20°C*

A culture of strain 15TP was grown at 20°C from A_{450} 0.12 to 0.4 with [^{32}P]P_i (2.0μCi/ml) present. The culture then received [^{3}H]uracil [15μCi (0.5μg)/ml] and samples (10ml) taken at intervals were poured on to crushed ice at −20°C. The cells from each sample were collected by centrifuging and lysates (final volume 0.5ml) prepared. A portion (150μl) of each lysate was centrifuged for 165min at 234000*g* through a 5ml linear sucrose gradient. Times of ^{3}H labelling were (*a*) 1min, (*b*) 2min, (*c*) 3min, (*d*) 5min, (*e*) 7.5min, (*f*) 10min. ○, ^{32}P radioactivity; ●, ^{3}H radioactivity.

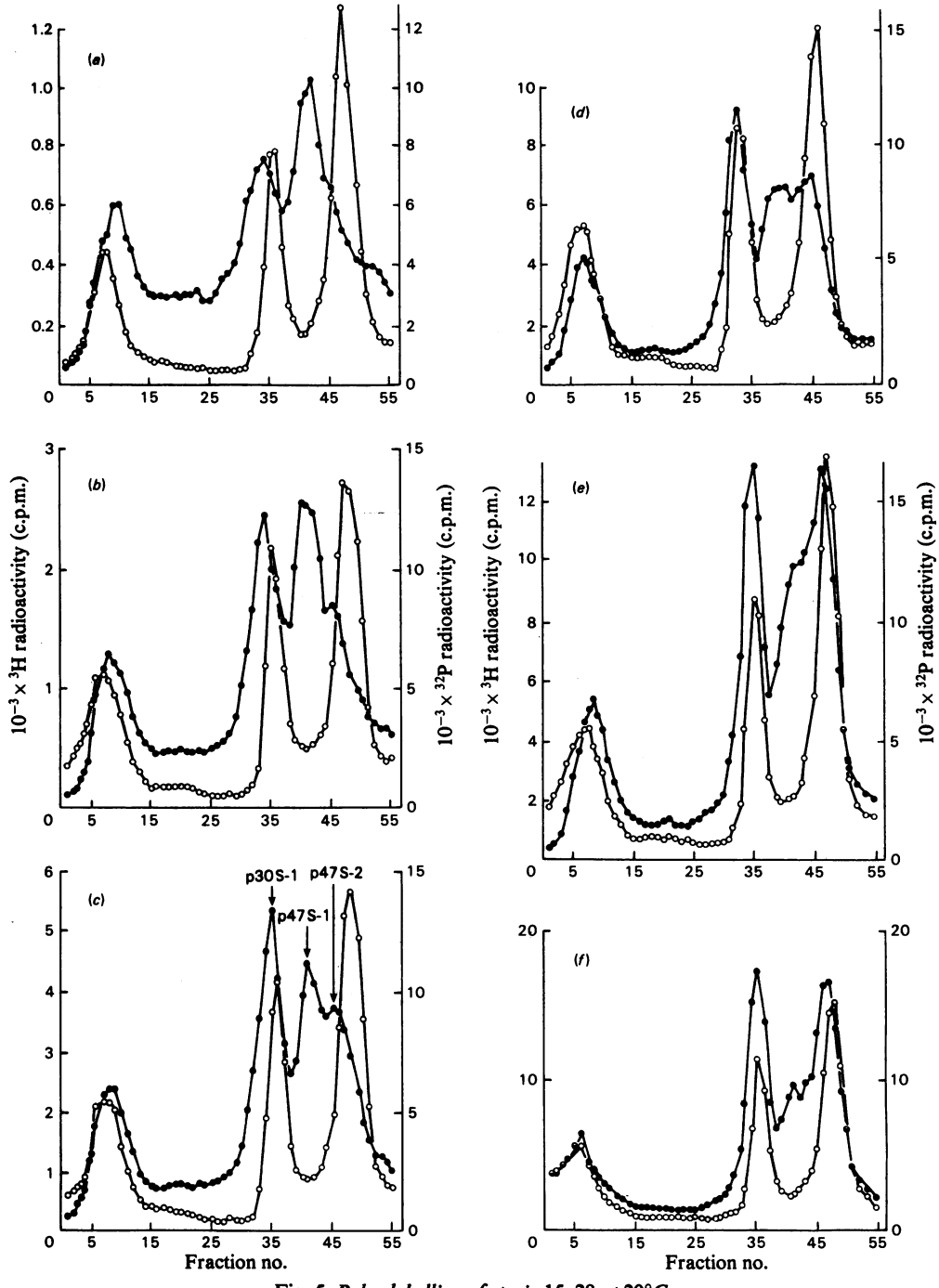

Fig. 5. *Pulse-labelling of strain* 15–28 *at* 20°C

A culture (70ml) of strain 15–28 was grown at 20°C from A_{450} 0.075 to 0.4 with [^{32}P]P_i (0.5μCi/ml) present. The culture then received [^{3}H]uracil [15μCi (2.5μg)/ml] and samples (10ml) taken at intervals were poured on to crushed ice at −20°C. The cells from each sample were collected by centrifuging and lysates (final volume 0.5ml) prepared. Portions (150μl) of each lysate were centrifuged for 4.5h at 234000*g* through 5ml convex exponential sucrose gradients. Times of ^{3}H labelling were (*a*) 5min, (*b*) 10min, (*c*) 20min, (*d*) 30min, (*e*) 40min, (*f*) 50min. ○, ^{32}P radioactivity; ●, ^{3}H radioactivity.

coincides with that of the ^{32}P-labelled 47S particles of the steady-state profile. The other major ^{3}H-labelled component in the pulse-labelling profile at early times ('p30S-1' in Fig. 2*a*: sedimentation coefficient about 26S) behaves similarly, in that after labelling for 30min or longer the ^{3}H radioactivity has virtually aligned with that of the ^{32}P-labelled 30S ribosomal subunits of the steady-state profile.

Various attempts were made to obtain better resolution of the pulse-label, particularly within the 'p47S' region. (*a*) The experiment of Fig. 2 used linear sucrose gradients. Sedimentation through convex exponential sucrose gradients (Noll, 1967) also failed to reveal discrete components within the p47S region, even when centrifugation had pelleted the 70S ribosomes. (*b*) Resolution was no better when labelling with [^{3}H]uracil was for shorter times. The sedimentation profile of organisms labelled for 40s (0.006 generation) showed a single broad p47S component. (*c*) For the experiments of Fig. 2, organisms were exposed to [^{3}H]uracil for increasing times. Similar results were obtained when, in 'pulse–chase' experiments, the mutant was labelled with [^{3}H]uracil for 1min and a 4000-fold excess of non-radioactive uracil then added. The sedimentation profiles of samples removed at intervals (Fig. 3) again show broad 'p30S-1' and 'p47S' components that apparently increase gradually in sedimentation coefficient as the chase is prolonged.

Ribosome assembly in strains grown at 30 *and* 20°C

Functional 30S and 50S ribosomal subunits can be reconstituted *in vitro* from their RNA and constituent proteins. In both cases, the rate of assembly is limited by conformational changes of high activation energy that partially formed ribosomes must undergo before more proteins can bind (Traub & Nomura, 1969*a*; Spillmann *et al.*, 1977). The extent to which assembly *in vitro* mimics the process in growing bacteria is arguable. But these considerations prompted an examination of synthesis in organisms grown at temperatures less than 37°C. Although strain 15–28 is slow-growing, it is not cold-sensitive; mean generation times (with those of the parent in parentheses) are about 180 (60) min at 30°C and 400 (140) min at 20°C.

When the parent strain was pulse-labelled at 30°C, sedimentation profiles were similar to those obtained after growth at 37°C, but with a lengthened time scale. This was also the case at 20°C; Fig. 4 shows that, with pulse times of 1–10min, precursors very similar to those observed at 37°C are visible in sedimentation profiles. At early times species p50S-1 is more prominent than p50S-2, whereas at times longer than 5min the situation is reversed. Pulse-labelling gives no information about the concentrations of precursor particles in exponentially growing bacteria; however, in no experiment at 20°C were components corresponding to p50S-1, p50S-2 or p30S-1 particles detected in either the ^{32}P-labelled steady-state profiles or the absorption traces. The

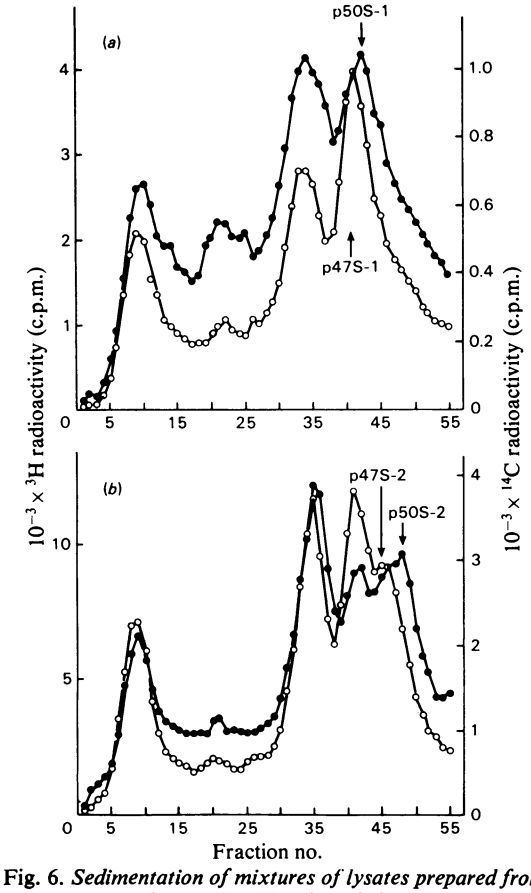

Fig. 6. *Sedimentation of mixtures of lysates prepared from strain* 15TP *and strain* 15–28 *pulse-labelled during growth at* 20°C

A culture (25ml) of strain 15TP was grown at 20°C from A_{450} 0.12 to 0.4. [^{3}H]Uracil [25μCi (0.2μg)/ml] was then added. At intervals (1min and 5min) after addition of the uracil, samples (12ml) of the culture were poured on to crushed ice at −20°C. The cells from these samples were collected by centrifuging and lysates (final volume 0.5ml) prepared from each. A culture (25ml) of strain 15–28 was grown at 20°C from A_{450} 0.06 to 0.4, when it received [^{14}C]uracil [1μCi (2μg)/ml]. Samples (12ml) were taken 5min and 20min later and lysates (final volume 0.5ml) made from each. Portions (200μl) of mixed lysates were centrifuged for 4.5h at 234000*g* through 5ml convex exponential sucrose gradients. The mixtures were: (*a*) 100μl of 15TP lysate (label for 1min) plus 100μl of 15–28 lysate (label for 5min); (*b*) 100μl of 15TP lysate (label for 5min) plus 100μl of 15–28 lysate (label for 20min). ●, ^{3}H radioactivity (strain 15TP); ○, ^{14}C radioactivity (strain 15–28).

steady-state profiles of Fig. 4 show that, at 20°C, the proportion of 70S ribosomes in extracts (relative to native subunits) is increased. This was consistently found, but not investigated further.

When strain 15–28 was grown at either 30°C or 20°C, neither the ^{32}P-labelling steady-state profiles nor the absorption traces revealed precursor particles (other than the 47S particles themselves). However, in experiments at 30°C, the broad 'p47S' peak (seen in sedimentation profiles after growth at 37°C) was rather asymmetric. Fig. 5 shows the results of an experiment for which growth was at 20°C. Centrifugation was through convex exponential gradients; polyribosomes and 70S ribosomes were sedimented. Growth of the mutant was so slow that exposure to [^{3}H]uracil was from 5 to 50min; even at the longest time, the ^{3}H- and ^{32}P-labelling profiles do not coincide. A single component that sediments more slowly than 30S ribosomal subunits is visible, together with two species that sediment between the 30S subunits and 47S particles. These latter are labelled 'p47S-1' and 'p47S-2' in Fig. 5; their approximate sedimentation coefficients are 38S and 43S. At 5 or 10min, p47S-1 particles predominate. As incorporation of [^{3}H]uracil continues, the components become comparable in the amounts of radioactivity that they contain, whereas at later times species p47S-2 is the major component and tends to merge with the newly made 47S particles.

Two experiments that compare the sedimentation properties of pulse-labelled components of strains 15TP and 15–28 are in Fig. 6. For these, both strains were grown at 20°C; the parent was pulse-labelled with [^{3}H]uracil and the mutant with [^{14}C]uracil. Portions of lysates were mixed and centrifuged. For Fig. 6(*a*), strain 15TP was labelled for 1min and strain 15–28 for 5min. These conditions permit comparison of the sedimentation of p50S-1 and p47S-1 particles in the virtual absence of p50S-2 and p47S-2 particles; the profiles show that p50S-1 particles sediment rather further than p47S-1 particles. This distinction is also apparent in Fig. 6(*b*), where the times of labelling (15TP, 5min; 15–28, 20min) were the shortest that satisfactorily resolve p50S-2 particles from p50S-1 and p47S-2 particles from p47S-1. The profiles show that p47S-2 particles sediment less far than p50S-2 particles.

Discussion

The sedimentation profiles obtained after pulse-labelling of the parent strain (15TP) during growth at 37°C are similar to those described for other 'normal' strains of *E. coli* (Hayes & Hayes, 1971; Lindahl, 1975). In particular, the two components p50S-1 and p50S-2 behave as expected of sequential ribosome-precursor particles. [Lindahl (1975) noted both these species together with a third precursor particle that cannot be distinguished from completed 50S ribosomal subunits by sedimentation and is not detected in the present experiments.] When growth of the parent is at 20°C, two very similar precursor particles are observed. It is rather surprising that these components are not now visible in steady-state profiles, because, if assembly *in vivo* involves precursor particles that undergo conformational changes of high activation energy, organisms grown at low temperatures should accumulate the precursors. Traub & Nomura (1969*b*) pointed out that extrapolation of the rate constant for the assembly *in vitro* of 30S ribosomal subunits to bacteria growing at 25°C would place about 50% of the cells' 16S rRNA in precursor particles. Similar considerations apply to the assembly of the larger subunit. Reconstitution systems must therefore differ, at least in efficiency, from synthesis in the intact cell.

The precursors to 50S ribosomal subunits that sediment more slowly than completed subunits probably account for about 0.5–1% of the 23S rRNA that the bacteria contain (Lindahl, 1975). The isolation and characterization of the proteins of these precursors (Nierhaus *et al.*, 1973) have therefore relied heavily on zonal centrifugation of extracts followed by the selection of appropriate regions of gradients for pooling and further concentration. The extent to which the more loosely bound proteins are lost during the extensive centrifuging needed to isolate the precursors is uncertain. However, comparison of the catalogues available (Nierhaus *et al.*, 1973) with the protein composition of 47S particles shows that the latter are unusual. The 47S particles are either a 'natural' ribosome precursor that the mutant accumulates, nor can they be regarded as a natural precursor with extra proteins added (Markey *et al.*, 1976).

The pulse-labelling experiments at 37°C suggest that the assembly of 47S particles is also unusual. As a protein-deficient, 'late' precursor of 50S ribosomal subunits, the 47S particles are in these respects similar to the p50S-2 precursor particles of the parent strain, although they differ from the latter in both sedimentation properties and composition (Markey *et al.*, 1976). From this viewpoint the p47S precursor particles in the mutant strain are then somewhat analogous to the p50S-1 particles of the parent. However, p47S particles appear in sedimentation profiles as a broad peak of radioactive material that apparently increases in sedimentation coefficient as the time of labelling is prolonged. The p47S material is presumably a mixture of species of different compositions and/or conformations that is labelled progressively. This obvious heterogeneity is not detected in the p50S-1 particles, although gradient centrifuging does not reveal whether these (and other precursor) particles are a mixture of species with a narrower range of sedimentation coefficients. An

alternative standpoint is that, because 47S particles are only slowly converted into 50S ribosomal subunits (Markey & Wild, 1976), the particles can, in effect, be regarded as the end product of synthesis. On this view, assembly in parent and mutant strains grown at 37°C is clearly different; the former involves two precursors and the latter only one intermediate whose sedimentation properties differ from those of the completed particles.

The results of the pulse labelling at 20°C suggest that the latter view is the more correct. Here, the behaviour of the mutant (but not the parent) strain alters. Two species (p47S-1 and p47S-2) are now resolved that behave as expected of sequential precursors to 47S particles. Presumably, in the mutant, some reaction in assembly has a rather high temperature coefficient and so becomes rate-limiting at lower temperatures of growth, but without causing the gross accumulation of precursor particles. In the mutant at 20°C, there are therefore three precursors to 50S ribosomal subunits (p47S-1, p47S-2 and 47S particles themselves) distinguishable by sedimentation; in the parent strain there are only two. If 47S particles are regarded as the major end product of assembly in the mutant, the p47S-1 and p47S-2 particles are 'equivalent' to the p50S-1 and p50S-2 particles of the parent strain. The sedimentation properties of the equivalent particles are similar, but the comparison made by centrifuging mixed lysates shows that they are not the same. A small difference in sedimentation could encompass large differences in composition, so that pathways of assembly in mutant and parent may diverge appreciably from their earliest stages.

On any view, then, the assembly of 50S ribosomal subunits by the mutant 15–28 differs markedly from that in the parent strain. Ribosome synthesis in the mutant merits further study as an example of the flexibility that cells retain in the synthesis of a precisely structured organelle.

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