Electron Microscopy of Polyribosomes Synthesizing Immunoglobulin Chains

COMPARISON WITH SIZES DETERMINED BY DENSITY-GRADIENT-SEDIMENTATION DATA

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Polyribosomes from the immunoglobulin-producing plasma-cell tumour X5563 were fractionated on a linear sucrose gradient and fractions containing polyribosomes carrying heavy (H) and light (L) immunoglobulin chains were examined with the electron microscope. The size distribution of the polyribosomes in the various fractions was determined. The results are consistent with previous estimates by Williamson & Askonas (1967) of the size of the polyribosomes synthesizing immunoglobulin chains, despite some discrepancies in their calculations based on sedimentation data. In particular, polyribosomes of up to 18 ribosomes were present in the part of the gradient containing H-chain polyribosomes. The polyribosomal clusters appeared to be true linear aggregates of uniformly spaced ribosomes, arranged mostly in open or zig-zag configuration. The appearance of most of them does not seem to support a general helical model of the polyribosome.

The heavy (H) and light (L) chains of the immunoglobulin molecule in normal lymphoid cells or plasma-cell tumours appear to be synthesized on polyribosomes of different sizes. Direct evidence has been obtained by sucrose-gradient centrifugation. It has been based on differences between the polyribosomal patterns obtained from tissues synthesizing different amounts of immunoglobulin or type of immunoglobulin chains (Becker & Rich, 1966; Shapiro, Scharff, Maizel & Uhr, 1966) or, more specifically, on precipitation of the fractionated polyribosomes with antisera against H and L determinants (Williamson & Askonas, 1967) or against immunoglobulin (Schubert, 1968).

Knowledge of the actual size of the polyribosomes forming H and L chains is important for the possible correlation between length of mRNA molecules and genes coding for immunoglobulin chains, but the few determinations are still affected by considerable experimental uncertainties, even in the experiments involving specific precipitation. The most explicit determination based on specific precipitation was made by Williamson & Askonas (1967), who estimated that H-chain polyribosomes contained 12–18 ribosomes and L-chain polyribosomes seven or eight. Their values were calculated from sedimentation data which may have been inaccurate, especially for H-chain polyribosomes (see the Discussion). It appeared, therefore, important to have an independent and more direct determination of the size of the polyribosomes carrying nascent

immunoglobulin chains by examination of the gradient fractions with the electron microscope. The present study was carried out under the same conditions of ultracentrifugation and fractionation of polyribosomes as those used by Williamson & Askonas (1967). The observations were mainly concentrated on the H-chain polyribosomes since the area of distribution of nascent L-chains was less well defined, owing to their poorer precipitability with antisera and the fact that L-chains represented only a small percentage of the total labelled proteins in this region. The results appear to be consistent with the previous estimates.

MATERIALS AND METHODS

Buffers. Solution E contained 1 mM-MgCl₂, 10 mM-KCl and 10 mM-tris-HCl buffer, pH 7.4. Solution G contained 10 mM-MgCl₂, 100 mM-KCl and 10 mM-tris-HCl buffer, pH 7.4.

Polyribosome preparation and fractionation. Purified polyribosomes were prepared from sodium deoxycholate-treated homogenates of X5563 plasmacytoma cells by sedimentation through a 2M-sucrose layer, as described by Williamson (1968). The polyribosomal pellet was resuspended in 0.1 M-sucrose in solution E (or, in few cases, G) and 1 ml of the suspension (about 3 mg of ribosomes) was layered on to 29 ml of a linear 10–30% (w/v) sucrose gradient in solution E (or G) and spun for 2½ h at 24 000 rev./min in the Spinco SW25 rotor. Fractions (1 ml) were collected by syphoning the contents of the tube with an Everett automatic syringe (Williamson & Askonas, 1967).

or by puncturing the bottom of the tube. In some experiments 1 ml of the supernatant (equivalent to 0.7 g of tissue) of a homogenate of tumour tissue treated with 0.5% sodium deoxycholate in solution E or G and spun at 200 g for 5 min was directly layered and analysed on the gradient. The tissue was homogenized in the presence of Macaloid (Williamson & Askonas, 1967) and a partially purified rat liver ribonuclease inhibitor (e.g. Northup, Hammond & La Via, 1967).

Electron microscopy. Several methods of preparation for electron microscopy were used; the polyribosomes were very fragile and in all cases it was necessary to handle them carefully and in the cold to avoid excessive break-down. (a) Of the fraction to be examined, 0.5 ml was diluted first one- or three-fold with sodium phosphate buffer, pH 7.4, to a final concentration of 0.025 M (in some experiments the buffer containing 10 mM-MgCl₂ and sucrose at a concentration approximately equal to that of the fraction), and then with an equal volume of 8% formaldehyde in 0.050 M-sodium phosphate buffer (with or without added sucrose). After 15–60 min droplets of the suspension were deposited on grids coated with a collodion-carbon film, the excess of fluid was removed and the grids were washed with water or solution E and either positively stained with 0.25–0.50% uranyl acetate at pH 6.0 (cf. Shelton & Kuff, 1966) or negatively stained with 2% uranyl acetate in water (pH 4.5) or 2% sodium phosphotungstate, pH 7.0. (b) Drops of diluted but unfixed polyribosomes were deposited on grids and, after removal of excess of fluid, the adsorbed polyribosomes were fixed with a drop of 4% formaldehyde in phosphate buffer, and washed and stained as described above. In several experiments (a) and (b) fixation and staining were performed by floating the grids on the surface of the appropriate solutions. (c) In a few cases, polyribosomes adsorbed on the carbon film were directly stained with uranyl acetate without previous fixation.

The results as regards the size distribution of the polyribosomes were similar with all three methods. Satisfactory preparations were obtained by pre-fixation in solution [method (a)] although the ribosomes in each cluster tended to clump together and overlap, making it more difficult to count them. Method (b) gave a better display of ribosomes with little overlapping. Polyribosomes prepared according to method (c) were well displayed and still grouped in definite clusters but they appeared slightly more degraded and the individual ribosomes in each cluster were more widely separated and connecting threads between them were rarely detectable. Electron micrographs were taken at a magnification of 16000–20000 with a Siemens Elmiskop I electron microscope (used by kind permission of Dr R. J. C. Harris and Dr R. R. Dourmashkin of the Imperial Cancer Research Fund, Mill Hill) and with an AEI EM6B electron microscope of the Clinical Research Centre Laboratories, National Institute for Medical Research, the latter equipped with an anticontamination device. Polyribosomal counts were performed on prints of positively stained specimens prepared by methods (a) and (b). Negatively stained preparations were satisfactory, but since individual polyribosomes tended to clump more easily and only irregular areas of the grid were covered by the stain, they were considered less suitable for good sampling. Clusters were counted as separated polyribosomes when the

nearest ribosomes were separated by a distance equal to or larger than a ribosomal unit (about 200 Å), even when their layout suggested the possible derivation from a single larger but broken polyribosome. Occasional clusters with contaminants or with extensively overlapping ribosomes were not counted, because of the possibility of artificial aggregation. These criteria were probably slightly biased against large polyribosomes in comparison with the distributions obtained with other techniques (e.g. metal-shadowed preparations).

RESULTS

A typical gradient of purified polyribosomes from X 5563 plasmacytoma cells used for one of the electron-microscopy preparations is shown in Fig. 1. The position of the monomer peak is clearly defined, whereas the polyribosome peaks are blurred and merge more or less completely into a large shoulder partly as a result of some mixing during the fractionation procedure. On the basis of precipitation experiments with specific antisera (Williamson & Askonas, 1967) fractions 4 and 5 were examined as representatives of polyribosomes carrying H-chains. Although they did not represent the upper limit of the distribution of H-chain polyribosomes, which extended to the bottom of the tube, they were used to avoid contamination with material of the pellet. For comparison and test of the size distribution of the polyribosomes in the gradient, fractions 13 and 14 were also examined. In this area polyribosomes with nascent L-chains were detected, although specific precipitation was spread over a larger region. In some cases fraction 23 (monomers) was examined to establish the extent of random aggregation of single ribosomes.

Plate 1 shows some representative fields from fractions 5, 13 and 23. The difference in size of the polyribosomes is clearly evident. However, it is

Fig. 1. Polyribosome pattern of one purified preparation of polyribosomes in solution G fractionated on a 10–30% sucrose gradient and used for electron microscopy. The arrows indicate the fractions (4, 5, 13, 14 and 23) selected for electron microscopy.

EXPLANATION OF PLATE I

Representative fields from fractions 5 (a), 13 (b) and 23 (c). Magnification $\times 60000$.

EXPLANATION OF PLATE 2

Examples of polyribosomes of 14 or more ribosomes present in fractions 4 and 5. Prepared according to methods (a) and (b) and positively stained with uranyl acetate or negatively stained with phosphotungstate (bottom left). Both linear (extended or curved) and zig-zag configurations are represented, often in the same polyribosome. A thin connecting thread between adjacent ribosomes is visible in some polyribosomes. Nominal magnification 100000 $\times$. The micrographs in the bottom row were taken with a microscope that had not been recalibrated and their actual magnification is probably higher.

Fig. 2. Distribution of polyribosomes according to size in (a) fractions 13 and 14 and (b) fractions 4 and 5. Results from fractions 4 and 5 and from fractions 13 and 14 were pooled since the distribution was not appreciably different in the adjacent fractions.

also evident from Plates 1(a) and 1(b) that some degradation of polyribosomes must have occurred during preparation as shown by the presence of single ribosomes or small clusters, which could not be expected to have sedimented to those parts of the gradient. I was unable to improve the results by small variations in the preparative procedure, such as, for example, slow and gradual dilution of sucrose and buffer, as suggested by Rich, Penman, Becker, Darnell & Hall (1963). In each fraction the ribosomes were grouped in clusters with a continuous distribution of sizes which extended up to a maximum size broadly characteristic of that fraction. The average distribution resulting from counting several representative fields of fractions 4 and 5 and 13 and 14 in one series of samples is shown in Fig. 2. Fractions 4 and 5 had 97.4% of ribosomes distributed in clusters of one to 18 ribosomes (with 27% between 11 and 18) and a peak at seven to nine ribosomes. Fractions 13 and 14 had only 1.7% of the ribosomes in clusters of 11 or more ribosomes, 96.1% distributed between one and eight (but only 11.8% between five and eight) and a peak at three to four. Fraction 23 (Plate 1c) was composed of single ribosomes with a few dimers.

The clear difference between the various fractions and the unaggregated appearance of the monomer fraction indicates that most of the clusters are unlikely to be formed by artificial aggregation of smaller groups during preparation. This is supported by the appearance of many of the individual clusters which favours their true polyribosomal nature. This point is illustrated in Plate 2 by some

examples of large polyribosomes (14–19 ribosomes) taken from fractions 4 and 5. The ribosomes are arranged either linearly, with adjacent ribosomes fairly uniformly spaced and sometimes connected by a thin dense thread, or in a more compact 'helical' (or more exactly, zig-zag) configuration. In several cases only a short segment of the polyribosomes is 'helical' and the remaining ribosomes are extended or irregularly arranged. 'Helical' arrangement was rather infrequent in preparations of purified polyribosomes, whereas it was relatively more common in fractions obtained directly from tissue homogenates treated with deoxycholate in solution G.

DISCUSSION

The purpose of the present study was to obtain an independent determination of the size of the polyribosomes carrying immunoglobulin chains to be compared with previous indirect estimates based on sedimentation data (Williamson & Askonas, 1967) which are uncertain, especially for large polyribosomes. Using the Martin & Ames (1961) approximation, Williamson & Askonas (1967) calculated values of about 300S for the H-chain polyribosomes and 180–190S for the L-chain polyribosomes and estimated that they corresponded to polyribosomes of 12–18 and five or six ribosomes, respectively. These estimates do not appear completely consistent with the available data on the relationship between the number n of the ribosomes and the sedimentation coefficient s_n . Data for mammalian polyribosomes are only available up to about $n = 10$ and in this range appear to be in reasonable agreement (especially if they are referred to the same value for the monomer): within the limits of the experimental errors, most of them appear to fit the relationship $s_n/s_1 = n^\alpha$ (Pfuderer, Cammarano, Holladay & Novelli, 1965), with $\alpha = 0.58$ (Gierer, 1963; Pfuderer *et al.* 1965; Breillat & Dickman, 1966; Compagnoni & Mahler, 1967); for mouse plasmacytoma polyribosomes Kuff & Roberts (1967) gave $\alpha = 0.62$. With $s_1 = 80S$, H-chain polyribosomes of 300S would have only nine or ten ribosomes, and L-chain polyribosomes four or five. On the other hand, the sedimentation coefficients estimated by Williamson & Askonas (1967) were only approximate because specific precipitation was not confined to a restricted zone of the gradient; those of H-chain polyribosomes were probably underestimated because precipitation extended to the bottom of the tube, where values higher than 300S would be expected, and also because in the lower part of the gradient the values calculated by using the Martin-Ames approximation are inaccurate and generally too low.

The electron-microscopy results show that a

considerable number of polyribosomes composed of 11–16 or –18 ribosomes (representing at least 27% of the ribosomes in fractions 4 and 5) are present in the lower part of the gradient, and suggest that the true upper limit of the polyribosome sizes in this zone is in this range. The broad size distribution prevents a more precise determination of this upper limit. The broadening is probably in part due to actual clustering and overlapping of polyribosomes of different sizes in the gradient, in part to some mixing during fractionation, but to a large extent to polyribosome breakdown during preparation for electron microscopy. This breakdown can be minimized by careful manipulation, but for large polyribosomes is never completely eliminated (see also Rich *et al.* 1963). In this case it was perhaps favoured by the use of deoxycholate, which removes some 25% of the protein present in untreated polyribosomes (Kuff & Roberts, 1967), possibly increasing their fragility during subsequent manipulations.

The size of the H-chain polyribosomes deduced by electron microscopy is compatible with a value of about 15 ribosomes theoretically expected for a polyribosome synthesizing a complete H-chain (Williamson & Askonas, 1967). It is comparable with the size of the polyribosomes (up to 16 ribosomes) observed in thin sections of membrane of the endoplasmic reticulum of cells from the same plasmocytoma (de Petris, 1967) and with the size of 16–20 ribosomes assigned by Becker & Rich (1966) to a peak presumably corresponding to H-chain polyribosomes. It seems larger than the size of the H-chain polyribosomes in Schubert's (1967) experiments, which from the published pattern appear at most to be decamers.

The distribution of polyribosomes in fractions 13 and 14 is sharper and the peaks at four ribosomes (30.7%) with an appreciable number at five ribosomes (10.3%) is close to the value expected from sedimentation data ($s = 190S$). The polyribosomes of these fractions are only indicative of the order of magnitude of the L-chain polyribosomes, since specific precipitation of L chains was rather poor and not well localized in the gradient; their size is not therefore incompatible with the estimates of five to six ribosomes by Williamson & Askonas and with a theoretical value of about seven ribosomes. On the basis of specific precipitation, other authors have assigned to L-chain polyribosomes values of four (Schubert, 1968) and five to nine (Wust, 1968) ribosomes.

It should be noted that in the present preparation the electron-microscopic evidence does not support a general model of a helical polyribosome (Pfuderer *et al.* 1965; Shelton & Kuff, 1966). Most polyribosomes were not arranged in helical configuration, especially after purification. If most of them were

in this configuration before preparation for electron microscopy, this configuration can apparently be easily disrupted. The 'helical' polyribosomes on the supporting film (Plate 2) are actually in a close-packed, but relatively extended, planar zig-zag configuration which, if derived from helices with three or more ribosomes per turn, should have undergone considerable conformational changes. In cells of several tissues or unicellular organisms regular helical polyribosomes have been clearly demonstrated in thin sections (e.g. Behnke, 1963; Andersson-Cedergren & Karlsson, 1967; Weiss & Grover, 1968). Similar helices are not evident in sections of X5563 tumour cells. In these cells at least some of the polyribosomes, namely those attached to the membranes of the endoplasmic reticulum, are not arranged in helices. Whether they assume this configuration in solution and how this is subsequently affected by fixation and drying is difficult to assess. Some packing of ribosomes during preparation is not unlikely; the frequent zig-zag or circular ('rosette') arrangement of the ribosomes in polyribosomes (see, e.g., Shelton & Kuff, 1966) could simply represent two alternative close-packing configurations on a flat surface ('trans' and 'cis') of a string of asymmetrical particles connected by a short but flexible thread of mRNA. At variance with previous proposals of a helical model (Pfuderer *et al.* 1965), discussion of the hydrodynamic properties of polyribosomes in solution (Filson & Bloomfield, 1968) suggests a more open, intermediate configuration, possibly induced in a random configuration by very weak attractive interactions between ribosomes. I consider that, as regards the majority of polyribosomes, not only the present observations but, in general, most of the electron-microscopy evidence, is more in agreement with a looser and variable configuration than with a strictly regular helical arrangement, and that only in particular cells, or in particular classes of polyribosomes of a cell, could additional factors contribute to determine the formation of true helices. This view is supported by the observation (Monneron, 1969; Krishan & Hsu, 1969) that transient formation of structures identical with helical polyribosomes can be induced under suitable experimental conditions.

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