Studies on the Protein Moiety of Plant Ribosomes

ENUMERATION OF THE PROTEINS OF THE RIBOSOMAL SUBUNITS AND DETERMINATION OF THE DEGREE OF EVOLUTIONARY CONSERVATION BY ELECTROPHORETIC AND IMMUNOCHEMICAL METHODS*

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SUMMARY

The approximate number and molecular weight of pea seedling ribosomal proteins were determined by two-dimensional electrophoresis on polyacrylamide gels containing either urea or sodium dodecyl sulfate. The small (40 S) ribosomal subunit contained 32 to 40 proteins and the large (60 S) subunit 44 to 55 proteins. The great majority of ribosomal proteins were basic and had molecular weights between 20,000 and 30,000. At least two proteins of higher molecular weight were found in the large subunit. Structural homologies between ribosomal proteins of different origin were estimated by two-dimensional electrophoretic analysis, by immunoelectrophoresis, double-diffusion tests, and quantitative immunoprecipitation. A high degree of evolutionary conservation was found, by the above methods, among ribosomal proteins of several species of higher plants, whereas little homology was found between chloroplast proteins and cytoplasmic ribosomal proteins of the same plant. No homologies were detected between chloroplast ribosomal proteins and proteins extracted from ribosomes of either mitochondria, bacteria, or blue-green algae.

Prokaryotes and certain cytoplasmic organelles (chloroplasts and mitochondria) contain a "small" type of ribosome sedimenting in the 70 S range, while the cytoplasmic matrix of eukaryotic cells contains a "large" type of ribosome sedimenting in the 80 S range (2-4). These two classes of ribosomes (prokaryotic and eukaryotic) differ from one another in several properties, even though they can exist within the same cell. Furthermore, important physical differences exist, within the eukaryotic class, between plant and mammalian ribosomes. The most striking of these differences is an increase by 0.65 to 0.70 \times 10^6 daltons of the large subunit of higher mammalian ribosomes as compared to the large subunit of higher plant ribosomes (5-7). Regardless of their origin, however, all the ribosomes studied so far perform the same basic functions and show a common pattern of organization.

They are composed of two unequal subunits, each of them containing several different protein molecules and either one or two (in the case of the large subunit) RNA molecules.

The development of disc gel electrophoresis (8, 9) and two-dimensional electrophoreses on slabs of polyacrylamide (10) has made possible substantial progress in the identification, purification, and enumeration of the individual ribosomal proteins. As a result of these studies, it is now clear that Escherichia coli ribosomes contain 55 different proteins (11-13) while mammalian and yeast ribosomes contain approximately 70 and 80 proteins, respectively (14-19). No information, however, is available concerning the number of proteins in ribosomes and ribosomal sub-units of higher plants.

The proteins extracted from the ribosomes of several species of either bacteria, plants, or animals have been found to be more or less species-specific (20-27) in spite of an apparent conservation of their size within each group (15, 28). These studies, however, were carried out by methods (either column chromatographic separation or one-dimensional electrophoretic analysis) which do not allow a complete separation of all of the protein components of the ribosome. For this reason, the actual degree of species specificity among ribosomal proteins of different organisms (20-25) as well as the degree of dissimilarity between chloroplast (21, 25, 29) or mitochondrial (30) and cytoplasmic ribosomal proteins from the same cell was likely to be underestimated. Recently a number of bacterial ribosomal proteins have been characterized also by two-dimensional gel electrophoresis (11, 31, 32).

In the present study we have examined the problem of species specificity among the ribosomes of different plant species using both two-dimensional electrophoretic analysis and immunochi-chemical methods. In addition, the recent development of a simple method for preparing relatively large quantities of small and large subunits of plant ribosomes which are potentially active in protein synthesis (5-7) has now enabled us to determine the approximate number and molecular weights of the proteins extracted from sub-units of plant cytoplasmic ribosomes.

MATERIALS AND METHODS

Buffers

The following buffers were used throughout this study: Buffer A (30 mm Tris-HCl (pH 7.4), 6 mm 2-mercaptoethanol, 10 mm MgCl₂, 0.5 M NH₄Cl); Buffer B (50 mm Tris-HCl, pH 7.8, 6 mm 2-mercaptoethanol, 14 mm MgCl₂, 60 mm KCl); Buffer C (20 mm Tris-HCl (pH 7.6), 4 mm magnesium acetate, 1 mm dithiothreitol, 3347
Preparation of Cytoplasmic, Chloroplast, and Bacterial Ribosomes

Crude (unwashed) cytoplasmic ribosomes were obtained from young leaves of beans, peas, spinach, and from wheat germ as previously described (25). Washed ribosomes were obtained by resuspending the crude ribosomes (at a concentration of approximately 5.0 mg per ml) in Buffer A. The ribosome suspension was left overnight at 0-2°C in this buffer and then centrifuged for 45 min at 16,000 rpm at 2°C in a Sorvall centrifuge. The supernatant fluid so obtained was centrifuged for 3 hours at 48,000 rpm in the Spinco 50Ti rotor through a 2.0 ml cushion consisting of 2 M sucrose in Buffer A. The resulting ribosomal pellets were dissolved in Buffer B at 5 to 10 mg per ml and stored at -80°C. Bean and spinach chloroplast ribosomes were prepared as previously described (25) but they were not washed in high salt, since this procedure causes extensive degradation of these particles. E. coli ribosomes were prepared according to Salas et al. (33), while Bacillus stearothermophilus and Bacillus subtilis ribosomes were prepared as described by Geisser et al. (31). Ribosomes of the monocellular blue-green alga Anacystis nidulans were kindly provided by Dr. W. Zillig (Max-Planck-Institut für Biochemie, München). Mitochondrial ribosomes of Neurospora crassa were a kind gift of Dr. H. Küntzel (Max-Planck-Institut für Experimentelle Medizin, Göttingen).

Preparation of Plant Cytoplasmic Polysomes and Ribosomal Subunits

Plant polysomes were isolated from 2-day-old pea seedlings, as previously described (5). The plant leaves were dissected and homogenized for approximately 1 min with quartz sand in an ice-cold mortar. The paste so obtained was extracted with 2 to 2.5 volumes of Buffer C containing 0.5 M sucrose. The homogenate was filtered through several layers of cheesecloth and centrifuged for 20 min at 15,000 rpm in the Sorvall centrifuge to yield a yellowish opalescent postmitochondrial supernatant. After addition of Triton X 100 to a final concentration of 0.5%, the postmitochondrial supernatant was centrifuged at 15,000 rpm for 20 min and the supernatant fluid so obtained was loaded on top of a discontinuous sucrose gradient consisting of 5.0 M of 2.0 M sucrose in Buffer C as the bottom layer and 7.0 ml of 0.7 M sucrose in Buffer C as the top layer, and centrifuged for 24 hours at 2°C and 27,000 rpm in the SW 2 Ti Spinco rotor. The polysome pellets so obtained were stored at -80°C. For the preparation of ribosomal subunits, the previously described procedure (7) was scaled up and slightly modified in the following respects: Pea run-off ribosomes were produced by incubating 200 mg of polysomes in 35.0 ml of Buffer C containing 35.0 µmoles of ATP, 15.0 µmoles of GTP, 350 µmoles of phosphoenolpyruvate, 750 µg of pyruvate kinase, and 6.0 ml of rat liver high speed postribosomal supernatant (non-dialyzed) (34). After 55 min of incubation at 37°C, the NH₄Cl concentration of the reaction mixture was brought to 0.3 M by addition of 4 M NH₄Cl, and the mixture was layered onto an 800 ml 7.4 to 38.9% (w/w) hyperbolic sucrose density gradient (35) containing 10 mM potassium phosphate buffer (pH 7.4), 1 mM magnesium acetate, 6 mM 2-mercaptoethanol, and 0.30 M NH₄Cl. After 17 hours of centrifugation at 20,000 rpm in the B XV Ti zonal rotor, the gradient was pumped through the flow cell of a Beckman DB spectrophotometer and fractions of 15 ml were collected. Fig. 1 shows the absorbance profile of a typical separation of pea ribosomes into subunits. The indicated fractions (cf. legend of Fig. 1) were pooled and the subunits therein recovered by centrifugation for 18 hours at 50,000 rpm in the Spinco 60 Ti rotor.

Extraction of Ribosomal Proteins

Ribosomal proteins were extracted from ribosomes and ribosomal subunits by either the LiCl-urea (36) (for most immunological studies) or by acidic extraction (37) (for electrophoretic analysis). The two methods have been shown to yield proteins with identical electrophoretic patterns (29).

Electrophoretic Methods

Two-dimensional polyacrylamide gel electrophoreses were always run at room temperature under three different conditions (see also “Results”).

Method A: Standard Two-Dimensional Electrophoresis—The procedure was identical with the one described by Kaltschmidt and Wittmann (10) with 8% acrylamide, pH 8.6, in the first dimension and 16% acrylamide, pH 3.0, for the second dimension. Electrohoresis times were 40 hours at 80 volts and 24 hours at 105 volts for the first and second dimension, respectively.

Method B: Modified Standard Two-Dimensional Electrophoresis—The origin was not at the midpoint between anode and cathode but was moved closer to the anode (5.0 cm from it). Urea was not present in either first or second dimension tray buffers. For the first dimension 4% acrylamide containing 6 M urea was used and the pH of the tray buffer was adjusted to 8.6 by addition of 55 ml of 2 N NaOH. The run time was 20 hours at 90 volts. For the second dimension 10% acrylamide (in 6 M urea) was used. The gel had a pH of 4.6 and the tray buffer a pH of 4.4. The run time was 24 hours at 58 volts.

Method C: Sodium Dodecyl Sulfate Two-Dimensional Electrophoresis—After the electrophoresis in the first dimension (identical with that of “Method B”) the gels were dialyzed at 45°C for 20 min against 500 ml of 0.1 M sodium phosphate buffer (pH 7.1) containing 7.5 M urea, 10 mM 2-mercaptoethanol, and 1% sodium dodecyl sulfate. This was followed by 500 ml of 0.02 M sodium phosphate buffer (pH 7.1) containing 7.5 M urea, 10 mM 2-mercaptoethanol, and 1% sodium dodecyl sulfite. Finally 400 ml of 0.01 M sodium phosphate buffer (pH 7.1) containing 7.5 M urea, 10 mM 2-mercaptoethanol, and 0.1% sodium dodecyl sul fate. For the electrophoresis in the second dimension, 10% acrylamide containing 6.0 M urea and 0.2% sodium dodecyl sulfate in 0.1 M sodium phosphate buffer (pH 7.1) was used. The gel was a 8% acrylamide slab gel containing 0.1 M sodium phosphate buffer (pH 7.1) containing 0.1% sodium dodecyl sulfate. The run time was 17 hours at 45 volts.

Preparation of Antibodies

Antisera against whole ribosomes and ribosomal subunits were raised in random bred rabbits by means of intramuscular and intracutaneous injections of antigens (38). Prior to onset of the immunization, nonimmune serum was taken from each animal. The injections were made on 10 different sites of the body according to the following schedule: the first injection was made on Day 0 and consisted of 5 to 10 mg of antigen in 3 ml of Buffer 1 and an equal volume of Freund’s adjuvant (Difco). Two other injections followed on the 14th and 21st day, respectively. Each injection contained the same amount of antigen and an equal volume of incomplete Freund’s adjuvant. On the 26th day the
animals were bled and the first immune serum was collected. The titers of the antisera were estimated by double diffusion test and by quantitative precipitation test. If the titers were low, or whenever they began to decrease significantly, boost injections were made. The animals were routinely bled from the ear vein after every 10th to 14th day. All the experiments were performed with antisera obtained from animals immunized for at least 3 months. The titers of the sera were found to be stable between 3 months and 2 years. The antisera were kept at -20° without any added preservative.

**Immunological Methods**

The Ouchterlony’s double diffusion tests and the quantitative immunoprecipitations were carried out as previously described (38). When whole ribosomes were used, the reactions were performed in two different buffers, D or E (cf. “Results”). When ribosomal proteins were used, they were extracted by the LiCl-urea method, because these proteins show a higher solubility.

**RESULTS**

**Electrophoretic Studies**

Remarks Concerning the Electrophoretic Methods—Method A for the two-dimensional electrophoresis of ribosomal proteins (see “Materials and Methods”) was originally developed by Kaltschmidt and Wittmann (10) for the analysis of bacterial ribosomal proteins. When applied to plant ribosomal proteins, this method gives a relatively good separation of the chloroplast ribosomal protein (cf. Fig. 7a) but does not allow a complete separation of the cytoplasmic ribosomal proteins, which are poorly separated from one another (cf. Fig. 7b). The number of the resolved proteins is considerably lower than theoretically expected and the majority of the proteins appears to be concentrated along an oblique line in the upper right-hand part of the gel slab. This is apparently due to the narrow distribution range of molecular weights and isoelectric points displayed by these proteins. Several attempts were made to improve the separation of the cytoplasmic ribosomal proteins by increasing the length of the electrophoresis or by decreasing the concentration of the gel in the second dimension. The best results were obtained when the conditions described under Method B (cf. “Materials and Methods”) were used. With this method the proteins were better resolved from one another and many of them migrated closer to the electrophoretic front (cf. Figs. 2 and 7).

**Examination of Pea Cytoplasmic Ribosomal Proteins by Two-Dimensional Polyacrylamide Gel Electrophoresis (Method B)—**The two-dimensional electrophoretic analyses Method B of the ribosomal proteins extracted from the small and large subunits and from the 80 S monomeric ribosomes of pea seedlings are presented in Figs. 2 and 3. As seen from the figures, the 40 S (Figs. 2a and 3a) and 60 S (Figs. 2b and 3b) patterns are grossly dissimilar from one another and, contrary to what is described in a previous report (29), in which the ribosomal subunits are produced with the aid of EDTA, very few or none of the ribosomal proteins seem to be present in both subunits. Accordingly, the 80 S pattern (Fig. 2c) seems to arise from the complementation of the patterns of the two subunits, although it is impossible to determine with certainty the origin of several proteins found in the 80 S ribosomes, due to the complexity of the pattern and to the fact that many proteins of the two subunits have similar electrophoretic mobility. Characteristic of the 40 S pattern is the circle of about 10 to 14 proteins seen near the center of the plate (Figs. 2a and 3a). Also noteworthy is the almost complete absence of acidic

![Fig. 2 (top). Two-dimensional electrophoretogram of pea seedling ribosomal proteins (Method B).](pea 40 S)

![Fig. 3 (bottom). Schematic representation of the electrophoretograms of the ribosomal proteins from the 40 S (a) and 60 S (b) ribosomal subunits of pea seedlings. Enumeration is from left to right and from top to bottom.](pea 60 S)
proteins in both subunit patterns (the anode side of the plates contains one clearly resolved protein and four faint spots in the case of the small subunits and only one faint spot in the case of the large subunits).

Examination of Pea Cytoplasmic Ribosomal Proteins by Sodium Dodecyl Sulfate Two-Dimensional Polyacrylamide Gel Electrophoresis Method C and Estimation of Their Molecular Weights—In order to gain information concerning the approximate molecular weights of the ribosomal proteins extracted from cytoplasmic ribosomes of pea seedlings, conditions were developed in which the electrophoresis in the second dimension was carried out in the presence of sodium dodecyl sulfate (cf. Method C of "Materials and Methods"). For the calculation of the molecular weights, a calibration curve was made (Fig. 4) in which the distances migrated by the ribosomal proteins of E. coli were plotted against the log of their known molecular weights (39). By comparison with this curve, it was possible to establish that the majority of the ribosomal proteins of both small (Fig. 5a) and large (Fig. 5b) subunits of pea seedling ribosomes have molecular weights comprised between 20,000 and 30,000. Two groups of proteins, homogeneous with respect to their molecular weights, can be recognized in the 40 S pattern. The first group consists of a set of approximately 6 to 8 proteins of about 30,000, while the second group contains a set of 12 or more proteins of about 20,000. Between the first and second group of proteins, about six spots can be seen corresponding to molecular weights intermediate between 20,000 and 30,000. Finally, 10 or more proteins displaying a higher electrophoretic mobility than the proteins of the second group are seen scattered in the lower part of the pattern. These proteins have molecular weights between 10,000 and 20,000. None of the proteins of the small subunit seems to have a molecular weight larger than 30,000 to 33,000. The large subunit, on the other hand, contains at least two proteins of 40,000 or more and very few proteins of less than 18,000 to 20,000. The great majority of 60 S proteins appears to be evenly distributed in the region of the electrophoretogram which corresponds to molecular weights between 20,000 and 30,000 (Fig. 5b). Finally, it should be noted also that the patterns obtained by two-dimensional electrophoresis on sodium dodecyl sulfate polyacrylamide gels clearly show (as did those obtained by electrophoresis with Method B of Fig. 2, a through e) that the large and small subunits of plant ribosomes are composed of entirely different protein molecules. The protein patterns of the small (Fig. 5a) and large (Fig. 5b) subunits are clearly different from one another. They are individually less complex than the pattern of 80 S monomers. This is even true when preseparated proteins from both subunits are mixed together and applied to electrophoresis (Fig. 5c).

Estimation of the Number of Ribosomal Proteins in Pea Seedlings—The enumeration of ribosomal proteins in complex electrophoretic patterns such as those presented so far is complicated because each electrophoretogram contains, along with the main spots, a certain number of faint spots and it is therefore very difficult to decide whether these faint spots correspond to true ribosomal proteins. An additional problem arises with spots having an irregular shape and possibly corresponding to more than a single protein. Similar results were also obtained with E. coli ribosomes (40).

Enumeration of the protein spots resolved in several electrophoretic runs similar to those in Fig. 2 (Method B) revealed the presence of 32 intensively stained and 8 faint spots in the small subunit pattern and 44 intensively stained and 11 faint spots in the large subunit. In the pattern of the 80 S monomers, on the other hand, approximately 83 spots were counted, 58 of which

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**Fig. 4.** Molecular weight scale determined with *Escherichia coli* ribosomal proteins. The logarithms of the molecular weights of individual *E. coli* ribosomal proteins are plotted against the distances migrated by each protein (from the origin line of the second dimension electrophoresis to the front of each spot) in two-dimensional gel electrophoreses containing sodium dodecyl sulfate (Method C) identical with those of Fig. 5.

**Fig. 5.** Electrophoretograms of pea seedling ribosomal proteins by two-dimensional gel electrophoreses containing sodium dodecyl sulfate (Method C). a, 1.8 mg of protein from 40 S ribosomal subunits. b, 2.4 mg of protein from 60 S ribosomal subunits. c, 2.4 mg of protein from 60 S ribosomal subunits and 1.9 mg of protein from the 40 S subunits.
were intensively stained and clearly resolved. Similarly, the enumeration of the proteins resolved in the two-dimensional electrophoreses containing sodium dodecyl sulfate revealed that the small and large subunits contain 35 to 40 and 50 to 60 proteins, respectively. In view of the difficulties in counting mentioned above, however, it is obvious that the above numbers should be considered rough estimates rather than absolute determinations of the number of ribosomal proteins in plant ribosomes. As with E. coli ribosomes, only the purification of the individual protein components of plant ribosomes and ribosomal subunits will hopefully allow a more precise and reliable enumeration.

Comparison of Two-Dimensional Electrophoretic Patterns of Different Plant Species—When the proteins extracted from the ribosomes and the ribosomal subunits of other plant species were separated by two-dimensional electrophoresis, patterns very similar to those of Fig. 2 were obtained. As seen in Fig. 6, a through c, a large degree of similarity can be found between the electrophoretic patterns of the small subunits. Thus, all or nearly all of the proteins resolved in the 40 S pattern of pea seedlings (dicot) (Fig. 3a) can be matched with identically or very similarly migrating proteins resolved in the 40 S pattern of corn (monocot) (Fig. 6a). Also the patterns obtained with the large ribosomal subunits are similar in different plant species (Figs. 3b and 6, b and c) and an extensive correlation between individual proteins of different plant origin can be established easily.

Comparison of Two-Dimensional Electrophoretic Patterns (Method A) of Chloroplast and Cytoplasmic Ribosomes—Previous one-dimensional electrophoretic analysis of spinach chloroplast ribosomal proteins revealed the presence of approximately 20 bands in both small and large subunits (20); in addition, at least eight proteins of the chloroplast ribosomes were found to migrate to the anode at pH 8.3 (29). These results are basically confirmed by the two-dimensional electrophoretic analysis (Method A) of proteins extracted from 70 S (chloroplast) ribosomes of spinach leaves (Fig. 7a). As seen in the figure, at least 40 different proteins are resolved and approximately 11 to 12 of them are acidic, having migrated to the anode (left) side of the gel slab. Examination of the pattern obtained by Method A, with the cytoplasmic ribosomal proteins of the same plant (Fig. 7b) indicates that little resemblance exists between these proteins and those extracted from chloroplast ribosomes. The differences between the 70 and 80 S patterns are particularly evident if one looks at the anode side of the plate: very few cytoplasmic ribosomal proteins are present on this side, thus confirming a previous report that cytoplasmic ribosomes of spinach leaves contain very few acidic proteins (29).

Immunological Studies

Remarks Concerning Immunological Methods—Antisera against intact ribosomes contain essentially antibodies specific against ribosomal proteins and show only very weak activity against rRNA (41). When intact ribosomes are precipitated at high Mg²⁺ concentrations with their homologous antisera, all the rRNA is present in the precipitate (Fig. 8). If the Mg²⁺ is omitted from the incubation mixture, however, the RNA remains in the supernatant, while the same (or at least a very similar) precipitation of the proteins occurs (Fig. 8). Thus, from these data it can be concluded that, under the conditions used, the anti-RNA antibodies play no role in the quantitative immunoprecipitation and, at high Mg²⁺ concentrations the precipitation of the ribosomes can be easily quantitated by measuring the precipitated RNA. In this study both RNA and protein precipitations were always measured and the percentages of cross-reactions were calculated from the ratio of the homologous versus the heterologous precipitation at the respective maximum of either RNA or protein precipitated. Although a larger amount of antibody is necessary to reach a plateau of protein precipitation compared to RNA precipitation (cf. Fig. 8), the extent of cross-reactions calculated for both RNA and protein precipitation was always found to be in good agreement.

In addition to the quantitative immunoprecipitation, two other immunological methods (Ouchterlony's double diffusion and immunoelectrophoresis) were used in the present study. In each Ouchterlony's double diffusion experiment a series of double diffusion tests was performed in which the relative concentrations...
of antigens and antibodies were varied. In addition, whenever possible, the reverse of each experiment was carried out so that if the ribosomes of a given species were used as antigens in one test, the antisera against the same ribosomes were used in the reverse test. The same approach was followed for the immunoelectrophoreses.

**Degree of Cross-Reaction between Cytoplasmic Ribosomes of Different Plant Species**—In order to quantify the degree of species specificity among the ribosomal proteins of different plant species, equal amounts of cytoplasmic ribosomes extracted from beans, peas, spinach, tobacco, and wheat were incubated with increasing amounts of antiserum against bean 80 S ribosomes, and the maximum degree of immunoprecipitation obtained in each heterologous cross-reaction was compared with the maximum degree of precipitation obtained in the homologous system. The results of these quantitative immunoprecipitation tests are summarized in the histogram presented in Fig. 9. As evident from the figure, very strong similarities exist between all the cytoplasmic plant ribosomes tested. The degree of reciprocal precipitation was determined comparing the maximum amount of heterologous versus homologous precipitation.

With any of the three lines obtained in the homologous reaction showing a pattern of nonidentity. In the reverse experiment, when anti-80 S bean serum was developed against either 70 or 80 S bean ribosomes, three to four sharp precipitation lines were seen in the homologous reaction, while only one sharp and one to two very weak precipitation lines appeared in the heterologous system. Also in this experiment the precipitation lines obtained in the homologous system showed no identity with the lines obtained in the heterologous system. The stronger precipitation lines obtained in the heterologous system with anti-80 S bean serum is explained by a substantially higher titer of this serum as compared with the anti-70 S serum.

Similar results were obtained when spinach chloroplast 70 S ribosomes were compared with cytoplasmic 80 S ribosomes from the same plant. In this case, however, the lines interfered with each other as in a pattern of partial identity. Analogous results were obtained by immunoelectrophoresis: the 70 S chloroplast ribosomes showed some precipitation lines when developed against the serum directed against 80 S ribosomes from the same plant, but, at least in the case of beans, these lines differed in their position from the precipitation lines obtained in the homologous reaction.

In addition, no cross-reaction appeared between the chloroplast ribosomes of one species and the cytoplasmic ribosomes of the others. The cross-reaction between the chloroplast ribosomes of the two different plants (bean and spinach) was significantly smaller than between the corresponding cytoplasmic ribosomes (cf. "Discussion").

In the interpretation of the above data it should be kept in mind that an extensive sequence homology (up to 60%) between two antigens may exist even in the absence of a cross-reaction (42). In addition, since we have used whole ribosomes (a mixture of several antigenic determinants) as antigens, the presence of more than one precipitation line in the homologous as well as heterologous reactions is not surprising. Each precipitation line conceivable arises from a heterogeneous group of proteins having similar diffusion constants and reacting with specific antibodies. For this reason any definitive conclusion of the type identity versus the partial identity is made very difficult.

A schematic synopsis of all the qualitative results, obtained by either immunodiffusion or immunoelectrophoresis, is given in Table I.

The degree of immunological cross-reaction between chloroplast and cytoplasmic ribosomes was determined also by quantitative immunoprecipitation. Equal amounts (300 μg) of salt-washed bean cytoplasmic (80 S) ribosomes were compared with increasing amounts of antisera against either 80 or 70 S bean ribosomes. The titers of the two antisera were normalized by addition of 0.9% NaCl to the serum (anti-80 S) which originally had the highest titer. As seen in Fig. 10, the cytoplasmic ribosomes of beans were precipitated not only by their homologous antiserum, but also, to some extent, by the antichloroplast serum. The extent of this cross-reaction varied between 10 and 29% (cf. "Discussion").

In another experiment (Fig. 11), 70 or 80 S ribosomes from beans and 80 S ribosomes from wheat germ were incubated with increasing amounts of antisera against 70 S bean ribosomes. As expected, the strongest reaction was obtained between the anti-70 S serum and the corresponding antigens, while the bean 80 S ribosomes showed approximately 25% cross-reaction. This value is deduced by comparing the amount of RNA present in the precipitate of the homologous reaction with that (30 to 60 μg) precipitated in the heterologous reaction. Even assuming a
Table I

Synopsis of qualitative immunological cross-reactions between ribosomal proteins of different origin

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*a +++, strong cross-reaction.
*b ++, weak cross-reaction.
*c + , very weak or uncertain cross-reaction.
*d - , no cross-reaction.
*0, experiment not performed.

Fig. 10 (left). Quantitative immunoprecipitation of bean cytoplasmic ribosomes with antiserum directed against either cytoplasmic or chloroplast ribosomes of beans. Cytoplasmic ribosomes of beans (300 μg) were incubated with the indicated amounts of antiserum directed against bean cytoplasmic (O—O) or chloroplast (O—O) ribosomes.

Fig. 11 (right). Quantitative immunoprecipitation of bean (cytoplasmic and chloroplast) and wheat germ (cytoplasmic) ribosomes with antiserum directed against bean chloroplast ribosomes. Each reaction tube contained 500 μg of bean chloroplast (O—O), bean cytoplasmic (O—O), or wheat germ cytoplasmic (Δ—Δ) ribosomes and the indicated amounts of antiserum directed against bean chloroplast ribosomes.

10% contamination of chloroplast ribosomes in the preparation of cytoplasmic ribosomes, one would not expect more than 25 to 30 μg of RNA precipitate, should all the cross-reaction be attributed to a contamination of the cytoplasmic ribosomes with chloroplast ribosomes. On the other hand, the 80 S ribosomes of wheat germ, which are certainly not contaminated with chloroplast ribosomes, show approximately 15% cross-reaction with antiserum directed against bean chloroplast ribosomes (Fig. 11).

Immunological Cross-Reaction between Chloroplast Ribosomes and Ribosomes of Mitochondria, Bacteria, and Blue-Green Algae—In a series of Ouchterlony's double diffusion tests, bean or spinach chloroplast ribosomes were incubated with a wide concentration range of antisera against either ribosomal proteins or whole ribosomes of bacteria (E. coli, B. subtilis, and B. stearothermophilus). In none of these tests did a precipitation line appear. The same result was obtained when, in the reverse experiment, antisera against chloroplast ribosomes were incubated with bacterial ribosomes or ribosomal proteins. In the control experiment, however, when 70 S chloroplast ribosomes were tested against their corresponding antisera, two to four sharp precipitation lines appeared (Table I). In similar experiments mitochondrial ribosomes of N. crassa showed no cross-reaction with antisera against bacterial ribosomes or with antisera against chloroplast (beans and spinach) ribosomes. The ribosomes of the blue-green alga A. nidulans showed no cross-reaction with antisera against chloroplast or bacterial ribosomes. Identical results were obtained by immunoelectrophoresis (Table I). Quantitative immunoprecipitation is more sensitive than either Ouchterlony's double diffusion tests or immunoelectrophoresis. We therefore used this method to find out whether or not possible immunological correlations between chloroplast and bacterial ribosomes existed which might have escaped detection by the other two methods. Bean chloroplast ribosomes (250 μg) were therefore incubated with increasing amounts of antiserum directed against the ribosomes of different species of bacteria (E. coli, B. subtilis, and B. stearothermophilus). Since both RNA and protein in the precipitate never exceeded the control values obtained with nonimmune sera, it was concluded that no detectable immunological correlation exists between the chloroplast and bacterial ribosomal proteins.

Discussion

The data presented in this paper offer further support for the notion that cytoplasmic and chloroplast ribosomes represent two distinct entities. The proteins extracted from these two types of ribosomes yield, upon two-dimensional electrophoresis, complex patterns which show little resemblance to one another. In addition, the immunological tests carried out have shown that the immunological cross-reaction found between the proteins of these two types of ribosomes is small and significantly lower than the one found between cytoplasmic ribosomes of distantly related plants such as beans and wheat. Concerning the weak cross-reaction found between cytoplasmic and chloroplast ribosomal proteins, it is not unlikely that this reflects the fact that some of the proteins of chloroplast and cytoplasmic ribosomes have similar primary structures, since it is known that at least some of the chloroplast ribosomal proteins are synthesized in the cytoplasmic matrix (43), under direction of nuclear genes (44).

Some considerations however should be made: both types of
ribosomes (70 and 80 S) cannot be obtained without a small (5% or less) cross-contamination and since antibodies can be produced against antigens injected as contaminants representing as little as 1% of the main antigen, there is no a priori reason to rule out the possibility that at least part of the observed cross reaction might have its origin in a cross-contamination of the antigens used to immunize the animals. An additional problem arises from the fact that, due to their greater susceptibility to this treatment, it was impossible to wash the chloroplast ribosomes with buffers containing high salt concentrations. It is possible, therefore, that some protein of cytoplasmic origin might have been absorbed on the surface of the chloroplast ribosomes during the course of their isolation. In conclusion, although it is likely that chloroplast and cytoplasmic ribosomal proteins contain some common antigenic determinant, a positive answer to this question must wait until the individual proteins of the two ribosomes types have been purified.

Of particular interest seems to be the conclusion, which emerges from both two-dimensional electrophoretic analyzes and from immunological studies, that strong similarities exist between all plant cytoplasmic ribosomal proteins (50% cross-reaction between dicotyledons and monocotyledons). Noteworthy is also the fact that chloroplast ribosomes from different plants display less immunological similarities between one another than the corresponding cytoplasmic ribosomes. This agrees with an earlier report (21) that the degree of species specificity of plant ribosomes, as judged from the electrophoretic patterns of the ribosomal proteins, is far greater for the chloroplast than for the cytoplasmic ribosomes. Since only 20% cross-reaction was found between E. coli and B. subtilis ribosomes, one can conclude that the ribosomal proteins of bacteria display a much greater evolutionary divergence than those of higher plants. In the light of these results, it is not surprising that the prokaryotic chloroplast ribosomes show no immunological cross-reaction with the ribosomes of bacteria, blue-green algae, or mitochondria. In contrast to the latter finding are the results of Pigott and Carr (45) who have found extensive homologies between the primary structures of the rRNAs of Euglena chloroplast and blue-green algae. If not due to the difference in sensitivity of the techniques used, these results could be taken as a possible indication that, during the course of evolution, the rRNA has been more extensively conserved than the ribosomal proteins.

Functionally and structurally intact subunits of plant ribosomes exhibit particle weights of $1.5 \times 10^6$ and $2.4 \times 10^6$ for the 40 and 60 S subunit, respectively (6). Since the small subunit contains 54% and the large subunit 46% protein, it follows that the daltons of protein are $0.81 \times 10^6$ and $1.1 \times 10^6$ in the 40 S and in the 60 S subunit, respectively (6).

Our electrophoreses (Method B and C) show that, depending on whether or not the faint spots are considered, the small and large subunit contain between 32 and 40 and between 44 and 55 protein spots, respectively. Thus, if we assume that the plant ribosomal subunits are not heterogeneous with respect to their protein composition, and if we count only the dark spots, the average molecular weight of pea ribosomal proteins would be approximately 25,000 for both subunits. On the other hand, if the faint spots are included, the average molecular weight of the ribosomal proteins drops to 20,000 for both small and large subunits. The latter figure seems to be too small, however, when compared to the molecular weights we have estimated. This is especially true for the proteins of the large subunit, the majority of which seem to have molecular weights larger than 20,000. The contamination of the ribosomal subunits with extraneous proteins and, in the case of the large subunit, with dimers of the small subunit, could account for the higher numbers. The ribosomal subunits, however, were obtained in the presence of $0.3 \text{ M NH}_4\text{Cl}$, and we have shown that washing the pea ribosomes with buffer containing this salt concentration produces particles of homogeneous and constant density in CsCl (6). Further increase in the salt concentration does not cause any increase in particle density (6). It is therefore unlikely that the subunits contain any adventitious proteins. In addition, the 60 and 40 S patterns show no resemblance to one another. Furthermore, no protein spots are seen in the 60 S pattern having the same mobility as the strongest spots of the 40 S pattern; this argues against the possibility of attributing the "supranumerary" proteins to a contamination of the large particles with dimers of the small subunits. The ribosomes of E. coli are heterogeneous for their protein composition; some proteins are represented in less than one copy per particle (46, 47) and others are present in more than one copy per particle (47). At the present time, no similar information is available concerning the stoichiometry of plant ribosomal proteins. However, if the proteins appearing as faint spots in our two-dimensional electrophoreses are indeed ribosomal proteins, the possibility that the subunits of plant ribosomes are, to some extent, heterogeneous with respect to their protein composition, should be taken into serious consideration. If this is indeed the case, one could then speculate that the faint or very lightly stained spots arise from the "fractional" proteins while some of the most intensively stained spots could originate from "repeated structures."

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