The levels of initiation factors and other translational components were compared in crude lysates of Escherichia coli grown at different rates. Cells were grown in media containing [35S]sulfate and different carbon sources, and harvested during mid-exponential phase after about 10 generations of balanced growth. Initiation factors (IF), elongation factors (EF), and a number of ribosomal proteins were identified unambiguously in gel patterns following two-dimensional polyacrylamide gel electrophoresis. The molar concentration of each protein was calculated from measurements of the radioactivity in excised gel spots and knowledge of the sulfur content of each protein. We found that the ribosomal proteins and elongation factors were present in equimolar amounts except for L7/L12 and EF-Tu which were 4-fold and 5-fold more abundant, respectively, and that the levels of each protein increased in proportion to growth rate. These results are similar to ones obtained previously by other methods, and serve to confirm the validity of our method. We found that the levels of IF2a and IF3 also were approximately proportional to growth rate. We also measured the levels of all three initiation factors using a radioimmune assay, showed that the factors are present in equimolar amounts, and confirmed that their abundance increased in parallel with ribosomes. We conclude that initiation factor levels are coordinately regulated with one another and with ribosome and elongation factor levels.

Ribosome levels in exponentially growing Escherichia coli are determined by conditions of growth and are approximately proportional to the growth rate (1, 2). Most of the individual components of the ribosome are present in stoichiometric amounts and are synthesized at the same rate. Recent studies of the organization and expression of ribosomal RNA and protein genes indicate that coordinate expression involves numerous operons, some of which are regulated by feedback mechanisms at the level of translation (3, 4). The levels of elongation factors EF-G and EF-Ts are stoichiometric with ribosomes whereas EF-Tu is present in a 5- to 7-fold molar excess over ribosomes. The elongation factors also increase in level as a function of increasing growth rate (5-8), and thus their synthesis is coordinated with that of ribosomal components. The single copy genes for EF-G and EF-Ts and the two genes for EF-Tu are located in operons containing ribosomal proteins, but a precise understanding of the regulation of their expression is still lacking. Because ribosomal proteins and elongation factors comprise up to 50% of the cell's total protein (1), the synthesis of translational components utilizes a significant proportion of the cell's available energy. Precise control of ribosome levels and synthesis is therefore of great importance.

The initiation phase of protein synthesis is promoted by three proteins called initiation factors IF1, IF2, and IF3. We are interested in how the levels of these proteins relate to other translational components and how such levels are regulated. We developed two immunochemical methods suitable for determining their levels in crude cell lysates: a classical RIA (9) and a quantitative immunoblotting technique (10). We have shown that the three factors are present in approximately equimolar amounts at a factor/ribosome ratio of 0.15-0.20 each in cells growing in enriched medium. These results suggest that the three initiation factor genes are coordinately regulated with respect to one another, but by a mechanism which produces a lower abundance of protein than is found for elongation factors or ribosomal components.

In this work we ask whether initiation factor levels are regulated like other translational components as a function of growth conditions. In order to be able to compare initiation factors with elongation factors and ribosomal proteins, we employed the two-dimensional NEPHGE/SDS gel system of O'Farrell et al. (11) to resolve and quantify the levels of these proteins. This method has been used by Neidhardt and co-workers (12-14) to study a number of translational components. Our results indicate that initiation factor levels parallel ribosome levels and that the factor genes are coordinately regulated with those for other translational components.

**EXPERIMENTAL PROCEDURES**

Materials—IF1, IF2a, IF2b, and IF3 were purified from E. coli MRE600 as described previously (15). Pure EF-Tu and EF-Ts were obtained from Boehringer-Manheim and EF-G was a kind gift of James Bodley (University of Minnesota).

Cell Growth and Preparation of Lysates—E. coli strain MRE600 cells were grown at 37°C in a rotary shaker incubator in MOPS-minimal media (16) supplemented with 1% bactotryptone, 0.5% yeast extract, and 0.4% glucose; 0.4% glucose plus amino acids; 0.4% glucose; 0.4% succinate; 0.4% glycerol; or 0.4% potassium acetate. For radio-

*Portions of this paper (including part of "Experimental Procedures," "Results," and "Discussion," Figs 4 and 5, and Table 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-1859, cite the authors, and include a check or money order for $3.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
active labeling of protein, starter cultures were grown in the above media to an A600 representative of early- to mid-log growth (0.2-0.5), and an aliquot of each was diluted 1000-fold into 100 ml of medium containing 39 μM Na2SO4 (New England Nuclear, 645 Ci/mmol). At early- to mid-log the cells were cooled rapidly to 0°C with the addition of ice, collected by centrifugation and washed twice in wash buffer (10 mM Tris-HCl, pH 7.4, 10 mM MgCl2). Cell pellets were stored at ~70°C until used. Frozen cells were thawed rapidly and suspended (0.5 g of cells/ml of buffer) in wash buffer. The cells were sonicated 3 times at 25 watts for 30 s (Heat Systems-Ultrasonics Inc., Model W140). After sonication, DNase I (Worthington) was added to a final concentration of 50 μg/ml. The cell lysate was incubated for 15 min at 2°C and centrifuged at 16,000 rpm for 20 min in a Sorvall SS-34 rotor. The clear supernatant was removed and stored at ~70°C. Such lysates typically contained 10,000 cpm/μg of protein.

Protein concentrations in the lysates were determined by the method of Lowry et al. (17) by using bovine serum albumin as standard. Ribosome concentrations were calculated from total RNA measurements as follows. Total RNA was measured in aliquots of perchloric acid-extracted crude lysates by the orcinol method as described (18). Ribosome concentrations were calculated on the basis that ribosomal RNA comprises 80% of total RNA (19) and a mass of 1.8 × 10^6 daltons.

**NEPHGE/SDS-Gel Electrophoresis**—Cell lysate samples (80 μg of protein/gel) were prepared for electrophoresis by adding a freshly prepared solution of ribonuclease A (Sigma) to 20 μg/ml and EDTA to 10 mM and incubating for 10 min at 20°C. Solid urea (Schwarz/Mann; 1.2 mg/ml of sample) was added followed by an equal volume of sample lysis buffer according to O’Farrell (20). Samples were put onto NEPHGE tube gels (2 × 125 mm) prepared according to O’Farrell et al. (11) with pH 3-10 ampholytes (Bio-Rad), and were overlaid with 50 μl of overlay buffer (11). The gels were run at 400 V for 3 h, removed from the tubes, placed into 5 ml of SDS sample buffer (20) and immediately frozen. In the second dimension gel system (21), we employed a 10-18% acrylamide gradient gel. The gel (1.5 × 125 × 100 mm) was run at 100 V for 1 h and at 150 V until the bromphenol blue dye band reached the bottom of the gel. Gels were stained with Coomassie blue R-250 (Pierce) and destained in methanol/water/acetic acid (5:5:1).

**Measurement of Radioactivity in Gel Spots**—Gel protein spots were cut out of destained gels with a scalpel blade and placed in 40°C. The acetic acid was removed and the gel spots were crushed with a glass rod. A 30/1 toluene scintillant/Protosol mixture was added. The crushed spots were incubated at 37°C for 24 h in capped glass vials, cooled and counted twice in a Beckman L5-25S liquid scintillation counter. In order to obtain maximal detection of radioactivity, it is necessary to cap the vials and incubate for at least 15-20 h.

**RESULTS**

**Two-dimensional Gel System**—We sought a system of gel electrophoresis which could resolve initiation factors and other proteins involved in translation without prior partial purification of the proteins in cell lysates. The method of O’Farrell et al. (11), which involves in the first dimension followed by a second dimension in SDS, appeared most appropriate for separating both the low molecular weight basic proteins such as IF1, IF3, and many ribosomal proteins, and also the higher molecular weight acidic proteins such as IF2a, EF-G, EF-Tu, and EF-Ts. The NEPHGE/SDS method, along with equilibrium isoelectric focusing/SDS, have been employed by Neidhardt and co-workers (12-14) to study a large number of E. coli proteins. However, based on their gel patterns, the NEPHGE/SDS system does not always resolve well the small basic proteins.

In order to optimize the resolution of proteins, we analyzed moderate amounts of radioactive lysates of cells grown in the presence of [35S]sulfate. In addition, we found that specific conditions have to be met to assure that the proteins enter the gel and resolve optimally. First, the RNase and DNase digestion steps are critical. Without adequate digestion, many of the basic proteins bind to nucleic acids and either appear in reduced amounts or as smeared spots. Second, as pointed out by O’Farrell (20), small proteins smear or are eliminated if too much SDS-agar embedding gel is placed onto the first dimensional gel. By using a minimum amount of agar, sharply resolved low molecular weight proteins were obtained. Third, ampholytes from several manufacturers were tested but none was found to be completely adequate for resolving the low molecular weight basic proteins. Many different lots of ampholytes caused the proteins to streak, become deformed, or even be obscured by the migrating ampholytes. We chose the lot of pH 3-10 ampholytes which produced the most sharply focused elliptical spots and did not obscure the small proteins. Following the conditions described in detail under “Experimental Procedures,” we were able to resolve many of the soluble factors and ribosomal proteins. A representative gel pattern is shown in Fig. 1.

**Identification of Proteins in the Gel**—Identification of spots corresponding to ribosomal proteins was made by excising the [35S]-labeled proteins from the gel and analyzing their migration in a two-dimensional gel system designed for 70 S ribosomal proteins. Details of the procedure are given in the Miniprint.

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**Fig. 1.** Identification of translational factors and ribosomal proteins in crude lysate patterns following NEPHGE/SDS polyacrylamide gel electrophoresis. Cells grown in MOPS-amino acids were harvested in mid-exponential phase and lysed. The lysate (80 μg of protein) was fractionated on a NEPHGE/SDS gel as described under “Experimental Procedures.” Top, the Coomassie stained gel; bottom, same as the top, except spots are labeled as identified by procedures described in the text. The molecular weight scale was determined from known proteins in the pattern.
Identification of selected, well resolved spots are shown in Fig. 1. We confirm and extend the list of identified ribosomal proteins in this gel system published by Bloch et al. (13) and Phillips et al. (14). The results and a discussion of major differences in ribosomal patterns are given in the Miniprint.

The locations of initiation and elongation factors were determined by co-migration of microgram quantities of the purified factors with $^{35}$S-labeled lysates and are shown in Fig. 1. The positions of the initiation factors were confirmed by immunoblotting techniques as described elsewhere (27). In addition, confirmation of identification of gel spots and evidence for their purity were obtained by comparing the partial protease or CNBr fragmentation patterns of the proteins in the spots with those of purified preparations. Detailed procedures and supporting data are provided in the Miniprint. These results show that the three elongation factors, IF2a, and IF3 are adequately resolved from other proteins and their positions in the gel are consistent with those reported by Phillips et al. (14). However, in our gel system, IF2b occurs in multiple forms (27) and is not well resolved from other proteins. Analysis by the equilibrium isoelectric focusing gel method (20) also showed contamination in IF2p spots (27) (results not shown). The IF1 spot is well separated from ribosomal proteins and IF1 does not co-migrate with such proteins in the NEPHGE/SDS system (see Miniprint, Fig. 5). However, the putative IF1 spot in Fig. 1 is much more intense at high growth rates than expected on the basis of immunological analyses (9, 10). Analysis by CNBr fragmentation (Miniprint, Fig. 4) indicates that the spot contains IF1 and at least one other protein that resists CNBr digestion. Thus, only IF2a and IF3 can be resolved and analyzed by our gel system.

Measurement of Protein Levels—Bacteria were labeled by balanced growth for 10 generations in $[^{35}]$S-sulfate media containing MOPS/glucose/amine acids, and lysate proteins were fractionated on NEPHGE/SDS gels as described in detail under “Experimental Procedures.” Identified protein spots were excised from stained gels, radioactivity was measured, and the abundance of the proteins was calculated as described. Raw data are given in Table I (in the Miniprint) and molar ratios are reported in Fig. 2. Nearly all ribosomal proteins are present in equimolar quantities, i.e. each approximates the molar content of identified gel spots (Fig. 1). Exceptions are L7 and L12, which together are present at a level 4-fold above the average ribosomal protein, and S8, whose level is inexplicably low. Ribosomal protein S6 also is found in an equimolar ratio with the other ribosomal proteins, in conflict with the recent observation of its being in a 2:1 molar ratio (28). However, recently revised measurements have shown S6 to be in equimolar ratio with ribosomes. \(^3\)

Elongation factors EF-G and EF-Ts resemble ribosomal proteins in abundance, whereas EF-Tu is present in 4.7-fold molar excess. These results are in excellent agreement with values obtained in many other laboratories (5-8). The level of IF3, expressed as a molar ratio of IF3 to average ribosomal protein, is 0.19, in agreement with previous results with the RIA (9). However, the IF2a/ribosome ratio is substantially lower (0.09). This is due to measuring only the a form, which is known to comprise only about half the total IF2 in such lysates (27). On the basis of these results, we conclude that accurate measurements of protein levels can be made by this method.

Factor and Ribosome Levels versus Growth Rate—It is known that ribosomes and elongation factors are relatively more abundant in fast growing cells (1, 2). We wished to determine whether initiation factor levels remain constant as a function of growth rate or whether they increase along with ribosomes as the growth rate increases. \(E. coli\) MRE600 cells were grown at different rates in three media containing $[^{35}]$S-sulfate and various carbon sources. The cells were harvested at mid-exponential phase and lysed as described under “Experimental Procedures.” Lysates were analyzed by NEPHGE/SDS gel electrophoresis to measure the levels of selected ribosomal proteins, elongation factors, IF2a, and IF3. Molar amounts of these proteins/mg of total protein are plotted against the growth rate constant in Fig. 3A. The average values of 11 ribosomal proteins (dashed line) confirm that ribosome concentrations are approximately proportional to the growth rate (1, 2). Similarly, EF-Ts and EF-G change coordinately with ribosomes, whereas the EF-Tu/ribosome ratio drops somewhat at the highest growth rate measured, as seen previously by Furano (5). These results are consistent with numerous reports on ribosome and elongation factor levels in such cells (6-8).

Our results with IF3 and IF2a indicate that initiation factor levels also increase with increasing growth rate. Initiation factor levels are plotted in Fig. 3A at 5 times their values in order to bring the scale in the range of that for ribosomal proteins and elongation factors. IF3 increases to the same degree as EF-G and EF-Tu, but not quite so steeply as ribosomal proteins. IF2a levels also increase with increasing growth rate, although the change at slow growth rates is not pronounced. Independent confirmation of these results was obtained by analyzing nonradioactive cell lysates by the RIA method (9). The RIA method enjoys two advantages over the NEPHGE/SDS gel method: it can be used to determine factor levels in very rich medium and it is able to quantitate IF1 in addition to IF2 and IF3. As shown in Fig. 3B, the levels of all three initiation factors increase as the growth rate constant increases from 0.65 to 2.1. The slopes for the factors are somewhat less steep than that for ribosomes (determined from measurements of RNA), especially for IF3. Nevertheless, the general trend is clearly seen by both independent methods.

\(^3\)Bartsch, M., Rienhardt, P. and Subramanian, A. R., manuscript submitted.
**DISCUSSION**

We have employed the NEPHGE/SDS two-dimensional polyacrylamide gel method of O'Farrell et al. (11) to separate initiation factors and other translational components and to measure their levels in crude lysates of *E. coli*. Unambiguously identified the location in the gel of the three initiation factors, three elongation factors, and 21 ribosomal proteins. By developing a method to measure accurately their molar concentration in crude cell lysates, we were able to measure changes in the molar ratios of the identified proteins under changing physiological conditions. Using this method, we found that IF2a and IF3 levels increase with cell growth rate, while elongation factors and ribosomal proteins. By an independent method, a radioimmune assay, comparable results were found for IF2 and IF3, and IF1 was measured as well. Initiation factor levels therefore are coordinated both with one another and with ribosomal levels. Using immunoprecipitation of IF2, Krauss and Leder (29) showed a constant concentration of this factor in cells growing at different rates. The discrepancy with our results may lie in the interpretation of their data and the methods they employed. First, their IF2 levels are plotted quite low on the graph, leading to difficulty in analyzing the actual data. When the change of IF2 levels between growth rate constants of 0.58 and 1.38 is estimated using their data, there is at least a 2-fold increase in IF2 content. Secondly, they show the EF-G concentration increasing 6-fold over the same growth rate range, a much greater change than has been found by others (6, 8). This indicates that their method was not capable of measuring accurately the changes in protein levels in crude cell lysates obtained at different growth rates.

The increase in ribosomal concentration as a function of growth rate is due to relatively greater rates of synthesis of ribosomal RNA and proteins (1, 2). The promoters for ribosomal RNA synthesis are under metabolic regulation, whereas the promoters for some of the ribosomal protein operons are not (4). Instead, ribosomal protein synthesis is coordinated with ribosomal RNA synthesis by a mechanism called "autogenous regulation" (30). The constancy in initiation factor/ribosome ratios at different growth rates, albeit at less than stoichiometric amounts, suggests that initiation factor gene expression is coordinated with that of ribosomes. The gene for IF3 (infC) has been mapped at 38 min on the *E. coli* genome and is not contiguous with ribosomal proteins or translational factors (31). Expression of the *infC* gene apparently is not under autogenous regulation since IF3 levels are proportional to *infC* dosage and IF3 added to a cell-free DNA-dependent protein synthesis system does not specifically inhibit IF3 synthesis (32). The *infB* gene for IF2 has been cloned and mapped at 68 min (33). Strains carrying multiple copies of the *infB* gene in plasmids overproduce IF2a and IF2b. These results suggest, but do not yet prove, that initiation factor gene expression may be controlled primarily at the level of transcription. If so, the work reported here implies that the promoters for IF2 and IF3 genes are under metabolic regulation. In collaboration with the laboratory of Dr. Grunberg-Manago (Paris, France), we are using the cloned factor genes to study their regulation of expression. In addition, we are using the two-dimensional gel method to measure in vivo synthesis and degradation rates of initiation factors.

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**Fig. 3. Factor and ribosome levels versus growth rate.** A, cells were grown in MOPS-minimal media supplemented with [35S]sulfate and glucose plus amino acids (k = 1.28), glycerol (k = 0.60), or acetate (k = 0.15). Lysates were prepared and analyzed by the NEPHGE/SDS gel procedure, and proteins were quantitated as described in detail under "Experimental Procedures." Ribosome content was estimated by averaging (see Fig. 2) the level of 12 ribosomal proteins. Enlarged factors EF-Ts and EF-G are plotted on scale, whereas EF-Tu (---O) values are reduced 5-fold. Initiation factors IF2a and IF3 (O---O) are augmented 5-fold. The growth rate constant (k) = ln 2/doubling time (h⁻¹). B, cells were grown in MOPS-minimal media supplemented with bacitracin/proline/glucose/yeast extract (k = 1.94), glucose plus amino acids (k = 1.59), glucose (k = 0.90), or succinate (k = 0.67) as described under "Experimental Procedures." The cells were harvested in mid-exponential phase and lysed. Lysates were analyzed by an RIA described in detail elsewhere (9). Initiation factors IF1 (O), IF2 (all forms) (■), and IF3 (△) were measured and molar amounts calculated on the basis of molecular weights of 8,016, 115,000, and 20,668, respectively. Ribosomes (O---O) were calculated from total RNA measurements as described under "Experimental Procedures."
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Identification of ribosomal proteins - Ribosomal proteins from well resolved spots in the MEPIGE/PSI gel were identified by mass spectrometry and electrophoresis with ribosomal proteins. Referring to the procedures of Takegawa et al. (22) modified as follows. Stained gel spots containing ribosomal proteins were excised and dried under vacuum for 5 hrs, then rehydrated in 500 μl of TCA buffer (50 μl triethanolamine pH 8.0, 10, 85, 5, 375 μl water) for 1 hr. The gel was crushed with a glass rod and incubated at 37°C for 30 min and then at 0°C for 30 min in the presence of 10% TCA buffer. A 0.1 volume of 3 M potassium acetate was added and the solution was incubated at -80°C for 30 min. The protein precipitate was pelleted by centrifugation (10,000 g, 4°C). The supernatant was discarded, and the pellet was extracted sequentially with acetone-MCI, cold 0.5 M chloroform-acetone (1:1) for 30 min and cold acetone (4°C for 30 min). Proteins were partially digested in stock for 1 h. Electrophoresis was continued for 5 h at 100 V and the gel was stained with Coomasie blue. Dried and subjected to autoradiography.
Initiation factor and ribosome levels are coordinately controlled in Escherichia coli growing at different rates.
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J. Biol. Chem. 1983, 258:1954-1959.

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