ple, heavy local pumping of groundwater in one area may cause local catastrophic land subsidence elsewhere. A problem that man can partly solve is the elusiveness of groundwater and the difficulty of capturing it in karst regions. In interior karst regions groundwater tends to discharge as large springs that lead to freshwater rivers. In coastal karst regions, however, freshwater springs and rivers may be scarce; large volumes of fresh water move directly into the sea. The high and uneven distribution of permeability in karst regions makes this waste to the sea a serious problem because it deprives man and other life of the nourishment that water in the right places brings.

Attempting to salvage this fresh water that is wasting to the sea is not likely to have offsetting ecological problems. Yet, the delicate natural hydrological balances within a karst region can easily be disturbed, and advantages gained by altering the hydrology may be offset by various detrimental effects.

Changes in hydrological balances are not unique to karst regions, but karst regions are more sensitive than other regions and the counteracting problems may be especially severe. It is necessary to understand environmental relationships, particularly those involving hydrology, to determine whether some actions by man are warranted. As a result of increased studies evaluation of these hydrological problems is coming into better focus. There is a need for improved knowledge of this important subject, which can be applied to the development of carbonate rock terranes for human use.

Fortunately, we can take specific action to maintain and upgrade the ecology of carbonate rock regions without waiting for additional field data to be collected. Our review of some hydrological relationships indicates that the ecological problems have generic solutions. These relationships have patterns that can be cast in terms of rules and principles. For example, good circulation of subsurface water leads to appreciable solution of the rock, solution leads to overall high permeability, and high permeability tends to lead to a water table well below most of the land surface and to scarcity of water on the land surface. Such general relations and tendencies need to be combined with pertinent specific data for a particular region or area. For ecological evaluations, it would be foolhardy to treat each carbonate rock terrane solely on its own merits, and to collect masses of data before decisions on the ecology are made. The data available are sufficient to give good first-round approximations to the solutions needed. Successively better approximations can be made as more data are considered, but in no case do we need to wait until new data are collected.

Summary

Climate exerts a universal dominant influence on ecology, but processes of karstification have an equally high ecological influence in carbonate rock regions. Development of karst features depends greatly on the degree to which water containing carbon dioxide has been able to move on and through carbonate rocks and to remove some of the rock in solution. Distinctive features of many karst terranes include scarcity of soils, scarcity of surface streams, and rugged topography; less distinctive are the highly permeable and cavernous rocks, especially at the shallow depths. This high permeability gives rise to many practical problems, including (i) scarcity and poor predictability of groundwater supplies, (ii) scarcity of surface streams, (iii) instability of the ground, (iv) leakage of surface reservoirs, and (v) an unreliable waste-disposal environment.

Natural karst processes in some carbonate rock regions have caused a greater restriction in the development of biota than man can ever be suspected of causing.

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Assembly of Bacterial Ribosomes

In vitro reconstitution systems facilitate study of ribosome structure, function, and assembly.

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The structure of ribosomes is complex. There are two ribosomal subunits, named according to their sedimentation coefficients. In *Escherichia coli*, the smaller, 30*S* subunit consists of one 16*S* RNA molecule and about 20 protein molecules. The larger, 50*S* subunit consists of one 23*S* RNA molecule, one 5*S* RNA molecule, and about 30 to 35 protein molecules (*1–3*). The development in recent years of systems permitting the reconstitution of ribosomes in vitro has advanced considerably our knowledge of the structure, function, and assembly of these organelles. Because the reconstitution of 30*S* subunits was achieved first and because our knowledge of this subunit is much more extensive than our knowledge of the 50*S* subunit, I center my discussion on the 30*S* subunits and refer only briefly to the 50*S* subunits; other aspects of ribosome research have been discussed more extensively elsewhere (*1–4*).

Assay of Ribosomal Functions

The activity of ribosomes and reconstituted ribosomes is routinely assayed by means of cell-free polypeptide synthesizing systems that are directed by natural messenger RNA or by synthetic mRNA such as polyuridylic acid. In addition, several partial reactions are used to assess the activity of ribosomes and reconstituted particles. These reactions include the binding of formylmethionyl-transfer RNA directed by

natural mRNA or the initiation codon AUG (*5*), the binding of other aminoacyl-tRNA's directed by synthetic mRNA, the peptidyl transferase reaction, the GTP binding that is dependent on G factor, and the UAA binding that is dependent on R factor. Details of these partial reactions are described in several reviews on the mechanism of protein synthesis (*3, 6*).

Partial Reconstitution of Ribosomes

The first step in the analysis of ribosome structure was the development of a system for reconstituting ribosomes that had been partially disassembled. The studies that led to this development were conducted by Staehelin and Meselson (*7*) and by workers at my laboratory (*8*) and were based on the observation that about 30 to 40 percent of the proteins (split proteins) in ribosomes are split off during density-gradient centrifugation of ribosomes in *5M* cesium chloride. The functionally inactive nucleoprotein particles (core particles) that remain can then be mixed with the split proteins, at which time the splitting process is reversed and active ribosomes are again produced. This partial reconstitution reaction is rapid and relatively insensitive to experimental conditions; the reaction is complete within a few minutes at 37°C (*9*). In these respects, the partial reconstitution reaction is very different from total reconstitution that will be discussed later.

Of the seven proteins split from 30*S* subunits (see Table 1, column 1), five [S3(P3), S5(P4), S9(P8), S10(P6), and S14(P11)] were purified and characterized (*10–12*). That three of these proteins [S3(P3), S10(P6), and S14-(P11)] are essential for certain ribosome functions (*10*) has been shown by reconstituting particles from 23*S* core particles mixed with fractionated split proteins, the split protein to be tested being omitted from the reconstitution mixture. Such experiments are called single component omission experiments. Omission of any one of the three essential proteins results in nearly complete abolishment of polypeptide synthesis directed by synthetic as well as by natural mRNA, and of the binding of tRNA directed by mRNA. The presence of S5(P4) is required for full activity in protein synthesis. I discuss the effect of omitting S9(P8) later in this article. Because the two remaining proteins, S1(P1) and S2(P2), had not been purified, we were not able to examine them individually.

The protein S14(P11) is a fractional protein, as defined by Kurland *et al.* (*13*); one 30*S* particle contains only 0.5 molecule of this protein (*14*) (Table 1). The biological significance of this heterogeneity of isolated ribosome populations is still unknown. The partial reconstitution experiments (*10*) strongly suggest, however, that all functionally active particles must contain S14(P11). Therefore, the heterogeneity of the ribosome populations with respect to this protein, for example, probably means that the population contains both active and inactive particles, the inactive particles being devoid of S14-(P11). Stimulation of the activity of isolated ribosomes upon addition of extra ribosomal proteins (*13, 15*) is consistent with this interpretation.

Total Reconstitution of 30S Subunits

The development of a partial reconstitution system was only a first step toward the functional analysis of ribosomal components. Comprehensive analysis of ribosomal structure and function would require reconstitution of the organelles from free RNA and individual proteins. In late 1967 the 30*S* subunit from *E. coli* was reconstituted from 16*S* RNA and a mixture of unseparated 30*S* ribosomal proteins (*16*). These experiments established the concept that the information for the correct assembly of ribosomal particles is contained in the structure of their molecular components, and not in nonribosomal factors.

Subsequently, 30*S* ribosomal proteins were separated and purified by several groups of investigators (*17, 18*) and reconstitution from mixtures of 16*S* RNA and 21 purified proteins was demonstrated (*18–20*). We can now reconstitute 30*S* particles which have activity

Table 1. Proteins from the 30*S* subunits of *Escherichia coli* ribosomes [for nomenclature, see (*11*)]. Proteins involved in the rate-limiting temperature-dependent step are compared with those found in the isolated reconstitution intermediate (RI) particles and with the 21*S* particles that are assembled in vivo. Unit (0.8 to 1.0 copy per ribosome), marginal (0.7 to 0.8 copy per ribosome), and fractional (0.5 or less copy per ribosome) proteins refer to the stoichiometric designations of Voynow and Kurland (*14*). Data on S15(P10b), S12(P10), and S13(P10a) are not available. Proteins that split off during centrifugation in 5*M* CsCl are indicated by SP (split proteins); those that remain associated with RNA in the 23*S* core particles are indicated by C (*10*). The extent to which each protein is required for the first step in the two-step reconstitution process (see text) is shown in the fourth column by ++, strongly required; +, moderately required; ±, weakly required; −, not required (*20*). The presence of each protein in the isolated RI particles (the 21*S* particles in vitro) is indicated in the fifth column by ++, present; +, present in reduced amounts; −, absent or almost absent (*20, 43*). Both S11(P7) and S9(P8) are probably present, but it is difficult to differentiate between these two proteins by electrophoresis on polyacrylamide gel. The protein composition of 21*S* particles accumulated by some cold-sensitive mutants (*44*) is shown in the sixth column: ++, present in amounts comparable to those in 30*S* subunits; +, present in reduced amounts; ±, found only in some preparations; —, not detected. For RI*, see page 868.

Protein	Stoichiometry	CsCl treatment	Required for RI* particle formation	Present in isolated RI particles	Present in 21*S* particles in vivo
S4(P4a)	Unit	C	++	++	+
S8(P4b)	Unit	C	++	++	++
S7(P5)	Unit	C	++	++	+
S16(P9a)	Unit	C	++	++	+
S19(P13)	Fractional	C	++	+	+
S17(P9b)	Unit	C	+	++	+
S15(P10b)		C	+	++	++
S5(P4)	Marginal	SP	±	+	−
S11(P7)	Fractional	C	±	(++)	−
S9(P8)	Unit	SP	±	(++)	±
S12(P10)		C	±	−	−
S18(P12)	Marginal	C	±	++	±
S20(P14)	Fractional	C	−	++	+
S13(P10a)		C	−	++	+
S6(P3b,c)	Marginal	C	−	++	+
S1(P1)	Fractional	SP	−	−	−
S2(P2)	Fractional	SP	−	−	−
S3(P3)	Marginal	SP	−	−	−
S10(P6)	Marginal	SP	−	−	−
S14(P11)	Fractional	SP	−	−	−
S21(P15)	Fractional	C	−	−	−

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as high as the original 30*S* subunits in the several ribosomal functions tested. Thus, we believe that in the list of 30*S* ribosomal proteins shown in Table 1 we have not omitted any significant macromolecular component that has an important function in polypeptide synthesis. However, this does not necessarily mean that all proteins listed in Table 1 are genuine ribosomal proteins, especially since several of them are present in amounts less than a single molecule per particle in isolated ribosomal preparations [fractional proteins (*13*, *14*); see Table 1]. Our reconstitution experiments have shown that almost all of the proteins listed in Table 1 are, in fact, required for full activity of ribosomes in various functional assays (*10*, *18*, *20*). The main exception is S1(P1). Omission of S1(P1) from the reconstitution mixture does not cause a reduction in any of the ribosomal functions tested (*18*, *20*). This protein usually fails to become incorporated into the reconstituted ribosome under the conditions of the reconstitution reaction, which include the use of buffers having high ionic strength (*21*). Isolated 30*S* ribosomal particles contain only about 0.1 to 0.3 copy of this protein per 30*S* particle (*14*). Thus, S1(P1) might not be a true ribosomal protein, although the presence of S1(P1) in assay mixtures has been shown to stimulate certain ribosomal functions (*22*). The accurate definition of ribosomal proteins is therefore difficult (*1*, *2*).

Single Component Omission Experiments

Four possible roles can be considered for the components of the ribosome. (i) A given component may be essential for the ribosome assembly but not required for any function; once assembly has occurred the component may be removed without loss of function. (ii) A given component may be required indirectly for some ribosomal function because its presence maintains an active center in a proper configuration in the ribosome structure. (iii) A given component may be a part of the active center, playing a direct role in a given ribosomal function. (iv) A given component may be required both for the assembly of the ribosome and for ribosomal functions.

To assign a particular role to any one component by single component omission experiments has proved difficult; omission of a single protein usually affects several functions while a given function can be shown to require the presence of each of several proteins. Nevertheless, single component omission experiments have provided us with some useful information. For example, the omission of protein S12(P10) results in the production of particles which show a pronounced reduction in translational error frequency under several conditions, whereas the omission of protein S11(P7) results in particles which show a pronounced increase in translational error frequency (*18*). Although the actual mechanism responsible for translational errors is not known, each of these two proteins must have some unique role in this mechanism since none of the other proteins which could be tested affect the quency of translation errors (*18*, *23*, *24*).

We now believe that some proteins are required only for efficient assembly [role (i) above], but not for the efficient functioning of the assembled particles. In our earlier studies several proteins were found to be important for the assembly of 30*S* subunits. Omission of any one of these proteins produced particles sedimenting at 20*S* to 25*S* rather than at 30*S* under the standard reconstitution conditions employed (incubation at 40°C for 20 minutes) (*18*). As expected, these particles showed greatly reduced activity in all the functions tested. On this basis, proteins S4(P4a), S8(P4b), S7(P5), S9(P8), and a mixture (called P9 at that time) of S16(P9a) and S17(P9b), were called "assembly proteins." However, the question of whether these proteins are required only for assembly or both for assembly and for ribosomal function was not answered.

Recent experiments strongly suggest that both S16(P9a) and S17(P9b), and also possibly S9(P8), have roles in the assembly reaction, but do not participate directly in ribosomal functions (*20*). After conducting a series of single component omission experiments we compared the results obtained from the partial reconstitution system with the results obtained from the total reconstitution system. With the partial reconstitution system the omission of S9(P8) did not have much effect on the functional ability of the reconstituted particles (*10*), whereas omission of the same protein in the total reconstitution system produced particles sedimenting at about 25*S* with very weak activity in several ribosomal functions (*18*). This suggests that S9(P8) is important in providing a "core structure" during the assembly reaction, but has no direct functional role. Apparently, the 23*S* core particles obtained by CsCl treatment retain the core structure, although they have lost S9(P8). Hence, S9(P8) may be dispensable in the partial reconstitution system.

Reconstitution in the absence of S16(P9a), or in the absence of both S16(P9a) and S17(P9b), resulted in particles which varied considerably depending on the duration of incubation. Systematic studies showed that in the absence of S16(P9a) and S17(P9b) the rate of reconstitution is very slow compared to the complete system containing these proteins. Nonetheless, 30*S*-like particles, apparently with full activity, are eventually produced. Thus, the proteins appear to be required for assembly but not for function. In early experiments, in which the time of incubation for the reconstitution was short, an inactive intermediate 21*S* particle was the dominant product. Thus, it is possible that other proteins also play a role only in the assembly process, their absence from the finished ribosome structure not seriously affecting function.

These findings may help to explain the evolution of the present ribosomal structure with so many protein subunits. Of course, it is still possible that the postulated assembly-specific proteins, such as S16(P9a) and S17(P9b), have some unknown functions in the finished ribosome structure which cannot be detected in vitro by assays we have used.

Other Functional Analyses of Ribosomal Components

There are several other approaches to the functional analysis of ribosomal components. In one approach the ribosomes are altered by chemical means and the resultant functional alterations are correlated with the chemical alterations of the components. In another approach, mutants with altered ribosome functions are isolated and the components responsible for the altered phenotypes are identified. The first ribosomal protein to be identified in this way was S12(P10), which was altered by the mutation of a streptomycin-sensitive strain of *E. coli* to a streptomycin-resistant strain (*25*). Both approaches

are now being used in extensive studies of ribosomal functions in many laboratories (*26*), the reconstitution technique being used to identify the components altered by chemical means or by mutation (*1*).

In an approach similar to those above, the reconstitution technique is used to prepare particles containing a single protein (or RNA) component with a specific modification. With such ribosomal particles, the role of the modified component (and the modified portions of that component) in the ribosome function or assembly can be analyzed. Components modified either in vivo or in vitro by chemical or enzymatic means can be used for such experiments.

In another approach the technique known as affinity labeling, which is used to study the structure of active centers in many enzymes (*27*), has been used in studies of ribosome function by Cantor and his co-workers (*28*). These workers used *n*-bromoacetylphenylalanyl-tRNA, an analog of peptidyl-tRNA, and identified two proteins L2 and L27, in *E. coli* 50*S* subunits as the proteins situated at or near the site which interacts with peptidyl-tRNA (the P-site).

Functional Role of Ribosomal RNA

Reconstitution experiments have established that intact 16*S* RNA is essential for the assembly of 30*S* subunits (*16*). For example, 18*S* RNA from the smaller subunits of yeast and rat liver cytoplasmic ribosomes cannot replace *E. coli* 16*S* RNA in the reconstitution. An RNA prepared from bacterial 23*S* RNA with the size similar to the 16*S* RNA is also ineffective as a replacement (*16*). Yet, some 16*S* ribosomal RNA from distantly related bacterial species, such as *Azotobacter vinelandii* or *Bacillus stearothermophilus* can replace *E. coli* 16*S* RNA and form functionally active hybrid 30*S* particles with *E. coli* 30*S* proteins (*29*). Although 16*S* RNA's from these three different bacterial species have some common base sequences, large portions are different. Thus, the requirement for a specific base sequence in ribosomal RNA is not absolute. Perhaps only some portions of the 16*S* RNA are directly involved in specific interaction with ribosomal proteins, these regions having structures (primary or tertiary) which are identical or are very similar among bacterial species (*29*). These sites on 16*S* RNA are being studied by several workers.

The reconstitution system for the 50*S* subunit from *B. stearothermophilus* has also been investigated. For reconstitution to occur in this system, 5*S* RNA as well as 23*S* RNA is essential (*30*). The 5*S* RNA's from *E. coli* and *Pseudomonas fluorescens* are both as active as *B. stearothermophilus* 5*S* RNA (*31, 32*). Although the primary sequence of *B. stearothermophilus* 5*S* RNA is not known, the complete primary sequences of both *E. coli* and *P. fluorescens* 5*S* RNA's are known (*33*). The sequences of these two 5*S* RNA's are alike over only about two thirds of the molecules. In this case, therefore, about one third of the base sequence can be altered in suitable ways without any detectable loss of function. It is conceivable that many more bases could be altered without affecting function. *Bacillus stearothermophilus* 23*S* RNA can also be replaced by *Staphylococcus aureus* 23*S* RNA is this system (*41*).

Studies of 5*S* RNA modified by chemical means have also shown that bases at several positions in the 5*S* RNA molecule can be modified without loss of function, as analyzed by reconstitution of the 50*S* subunit from *B. stearothermophilus* (*31*). Because the elimination of the 3′-end nucleoside of 5*S* RNA or the chemical modification of the ribose moiety of this terminal nucleoside does not affect the activity of 5*S* RNA (*31*), it has been suggested that models of protein synthesis (*34*) that invoke peptidyl-5*S* RNA as an intermediate are invalid.

By contrast, chemical modification (for example, by nitrous acid or monoperphthalic acid) of 16*S* RNA indicates that only a few base alterations (per molecule) are sufficient to destroy completely the reconstitution activity of the molecule (*29, 35*). Thus a major portion of the base sequences (exposed to these reagents) of the RNA appears to be important. However, in these experiments the inactivation appeared to be caused by the formation of subunits with abnormal configurations.

That ribosomal RNA has a direct functional role as well as structural role has not been shown by any chemical modification experiments. Yet, several other types of experiments suggest that ribosomal RNA may also have a direct role. First, 30*S* subunits isolated from colicn E3–treated *E. coli* are inactive in polypeptide synthesis in vitro (*36*). The inactive 30*S* subunits are indistinguishable from the active subunits in their physical behavior and retain all the ribosomal proteins. Reconstitution experiments show that all proteins are functionally intact, and the alteration responsible for inactivation resides in 16*S* RNA (*37*). The alteration appears to involve a single nucleolytic event at a site about 50 nucleotides from the 3′-end of the 16*S* RNA (*37, 38*).

The second indication of a functional role for RNA comes from the analysis of kasugamycin-resistant mutants of *E. coli* (*39*). The ribosomes from these mutants are resistant to kasugamycin during polypeptide synthesis in vitro. The alteration resides in the 30*S* subunit (*40*). By chemical analysis and reconstitution studies, it was shown that the absence of a methyl group in the normal sequence, $m_2^6Am_2^6$ ACCUG (*5*), of 16*S* RNA is responsible for the mutant ribosome behavior (*39*). The importance of methylation of 23*S* RNA with respect to lincomycin resistance in *S. aureus* has also been shown by the reconstitution technique (*41*).

With the availability of techniques for determining RNA sequences, as well as the reconstitution assay technique, it is somewhat surprising that relatively few investigators have attempted to determine the possible direct roles of ribosomal RNA in protein synthesis.

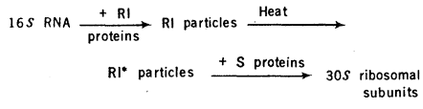
Mechanism of Assembly in vitro

Using individual purified 30*S* ribosomal proteins, we have studied the assembly of 30*S* subunits. Under the conditions of reconstitution, only seven of the proteins become bound to the RNA. Certain other proteins become bound only after some of the first seven proteins are bound (*19, 21*). [Other investigators, however, have presented results suggesting that only five out of these seven proteins are specific initial binding proteins (*42*).] The remaining proteins require the presence of proteins in both of the above groups in order to become bound. In this way, we have analyzed the sequence of addition of proteins to the 16*S* RNA molecule and have constructed the assembly map shown in Fig. 1 (*19, 21*).

The arrows connecting proteins to proteins in Fig. 1 represent only the associations that we detected during the experiments that were conducted to construct the map. Many other protein-protein interactions which stabilize ribo-

somal structure probably occur. In addition, it is conceivable that proteins other than the initial binding proteins also interact with 16*S* RNA in the finished ribosome structure.

It is probable that the "sequence" described in the assembly map corresponds at least approximately to the temporal sequence of assembly. Other evidence for this assembly sequence comes from the early kinetic studies of the assembly of 30*S* subunits which showed that there is a rate-limiting unimolecular reaction that requires a high activation energy (*43*). At lower temperatures, a subset of the 30*S* ribosomal proteins (called "RI proteins") interacts with 16*S* RNA, and intermediate particles (reconstitution intermediate, or "RI particles") accumulate. The isolated "RI particles" sediment at 21*S* in a buffer having a low concentration of magnesium ions; they are deficient in several proteins (called "S proteins" because they remain in the supernatant after 21*S* particles sediment), and have no functional activity. From this and other experiments done on the isolated "RI particles," the following reaction scheme was proposed (*43*):



In this scheme, the step, RI particle → RI* particle, represents the rate-limiting unimolecular reaction, and is thought to involve a large conformational change in the structure of the intermediate, the RI particle. The 21*S* RI particles discussed here should not be confused with various particles (20*S* to 25*S*) which accumulate when reconstitution occurs in the absence of one of the "assembly proteins" (see above). However, some 20*S* to 25*S* particles may have accumulated because of the effect on the same rate-determining step and they may be similar to the 21*S* RI particles.

We have attempted to determine the protein composition of the RI particle required for this presumed conformational change, that is, the RI proteins. First we determined the composition of the isolated RI particles (Table 1) (*20, 44, 45*). While some of the required proteins may have been lost during isolation, it is also possible that additional proteins become bound but are not required for the conformational change. Therefore, to determine which proteins must be present during the heating step in order to get the highest degree of reconstitution, we used a two-step reconstitution method (*20*). The 21 purified proteins were divided into two groups in various ways. One group of proteins was incubated with 16*S* RNA at high temperatures and, after cooling, the remaining proteins were added and incubated at low temperatures for a short time. In this way, we identified the proteins required during heating for efficient reconstitution to occur (Table 1) and observed some differences between the proteins identified in this way and the proteins found in isolated RI particles. First, S20(P14), S13(P10a), and S6(P3b,c), which are found in isolated RI particles, are unnecessary for the formation of RI* particles. These proteins can be added either before or after the presumed rate-determining conformational change has taken place, without any effect on the rate of reconstitution. Second, some proteins such as S12(P10), S19(P13), and S5(P4) are required for efficient RI* formation and yet are present only in small amounts or are not detectable in isolated RI particles. These proteins might constitute a part of the true RI particle that is simply lost during isolation, or they might aid in stabilizing the particles after the conformational change. Despite these observed differences, however, it should be emphasized that most of the proteins required for the formation of RI* particles are among those found in the isolated RI particles. Thus, the real intermediate particles that undergo the rate-determining conformational change must be similar to the isolated RI particles, with perhaps some additional proteins [such as S12(P10), S19(P13), and S5(P4)]. Proteins above the dotted line in the assembly map (Fig. 1) indicate the proteins found in the isolated RI particles and the additional three proteins mentioned above. Thus the incorporation of other proteins (under the dotted line) into the reconstituted 30*S* particles does appear to depend on the conformational change discussed above.

Several proteins have only a moderate or weak influence on the formation of RI* particles (Table 1). These proteins can be added after the heating step, with only a small decrease in the efficiency of reconstitution. It is probable that there are several different routes for assembly of 30*S* ribosomal subunits. Differences in free energy of activation among these several alternative routes may be rather small. Supporting this general conclusion is the striking observation that 30*S*-like particles with nearly full activity are slowly produced even in the absence of S16(P9a) and S17(P9b), both of which play major roles in the assembly process. It has not been determined whether such flexibility in the assembly process exists in vivo.

The tertiary structure of free 16*S* RNA and free 23*S* RNA is quite different from that within the ribosomes (*1, 46*). Thus, the RNA must undergo conformational changes during the assembly reaction. Some data suggest that the major change in ribosomal RNA (rRNA) takes place during the rate-limiting step (*20*); further investigations of the conformational changes in RNA are in progress.

As already mentioned, certain specific proteins are the first to bind to 16*S* rRNA (*21, 42*). Once properly formed, the RNA-protein complexes do not dissociate during subsequent assembly steps (*21*). Investigators in several laboratories are attempting to identify the sites on the 16*S* rRNA which bind these proteins (*47, 48*). For example, Zimmerman *et al.* have obtained several RNA fragments which interact with one or several of the initial binding proteins (*48*). From the chemical analysis of these RNA fragments, the approximate position of the binding sites for several initial binding proteins has been located on the 16*S* rRNA (*48*). It is noteworthy that five of the initial binding proteins [S4(P4a), S8(P4b), S13-(P10a), S15(P10b), S20(P14)] bind to the 5′-terminal 900 nucleotides of the RNA and only one, S7(P5), attaches to the 3′-terminal third of the molecule. Such studies are certain to produce useful information both on the structure of ribosomes and on the mechanisms by which the proteins recognize specific RNA structures.

Three-Dimensional Structure

Although x-ray or electron microscopic methods may eventually prove useful in the complete elucidation of the three-dimensional structure of ribosomes, these techniques have provided little useful information so far. This is due both to the lack of techniques for making ribosome crystals suitable for x-ray methods and to the asymmetry and complexity of ribosome structure. Most investigators now study three-dimensional structure by chemical methods, such as the use of bifunctional cross-linking reagents that reveal relationships among neighboring protein

components. The relationships among proteins that become bound, as revealed in the assembly map of 30*S* subunits (Fig. 1) may also reflect topological relationships among ribosomal proteins in the ribosomal structure (*21*). Thus, results obtained by chemical and other methods can be compared with the relationships shown in the assembly map. For example, Craven and his co-workers examined the protein components of subparticles produced by mild ribonuclease digestion of *E. coli* 30*S* subunits (*49*) and found that those proteins that cluster together in such subparticles are, in general, the proteins which show interrelationships in the assembly map. Similar results were obtained by Cox and his co-workers (*50*). The striking correlation between the assembly map and the results obtained by chemical studies supports the suggestion that the assembly map reflects the topological relationships of ribosomal proteins.

In most chemical studies of ribosome structure it is assumed (i) that the ribosomal preparation contains a homogeneous population of active ribosomes and (ii) that the ribosome structure remains intact during the chemical treatment used. In fact, only a fraction (at most 50 percent) of the ribosomal particles in most ribosome preparations is active and several proteins [fractional proteins (*13, 14*)] are actually missing from some (presumably inactive) ribosomal particles. Moreover, assumption (ii) is usually difficult to prove. Therefore, the information obtained by chemical methods on the three-dimensional arrangement of proteins within the ribosome structure should still be treated with caution.

Cross-linking reagents are currently employed by several investigators for the study of ribosome structure. For example, using tetranitromethane, Shih and Craven showed cross-linkage of S18-(P12), S11(P7), and S21(P15) which are interrelated on the assembly map (*51*). Traut and his co-workers, as well as several other groups, used the bifunctional reagent, bis-methyl suberimidate, and obtained results that do not correlate well with the assembly map (*52*). Some of the relationships shown among neighboring proteins in these studies may include protein-protein interactions that have not been revealed during the construction of the assembly map.

Some investigators have been studying the reactivity of 30*S* ribosomal proteins toward other chemical reagents, enzymes, or antibodies in the hope that relatively exposed proteins might be distinguished from relatively unexposed proteins within the ribosome structure (*53*). These studies have given somewhat conflicting results so far. Obviously, the results may vary depending on the kind of reagents used. In addition, as noted above, some of the reagents used may themselves have caused changes in the ribosome structure.

Several other techniques are being considered for use in the study of ribosome configuration. One is the use of fluorescent probes as molecular measures of distance [singlet-singlet energy transfer; see (*54*)] between proteins in the ribosomes. In preliminary experiments Cantor and his co-workers (in collaboration with us) have found that several 30*S* ribosomal proteins covalently labeled with suitable fluorescent dyes can be incorporated into functionally active reconstituted 30*S* subunits. Thus it appears to be possible, at least with some protein combinations, to measure the distance between two different ribosomal proteins (or between a protein and a particular position on 16*S* rRNA, for example, 3′-end) within functionally active 30*S* subunits.

Similarly, distances between proteins can be measured by neutron diffraction. Because the neutron-scattering properties of hydrogen are different from all other normally encountered atoms including deuterium, it is theoretically possible to measure the distance between two hydrogen-rich proteins in reconstituted ribosomes that otherwise contain heavily deuterated molecular components (*55*). If the distances between enough pairs of proteins can be measured by these techniques, it should be possible to determine the unique arrangement of proteins in the ribosome. In addition, the use of functionally active proteins labeled with fluorescent

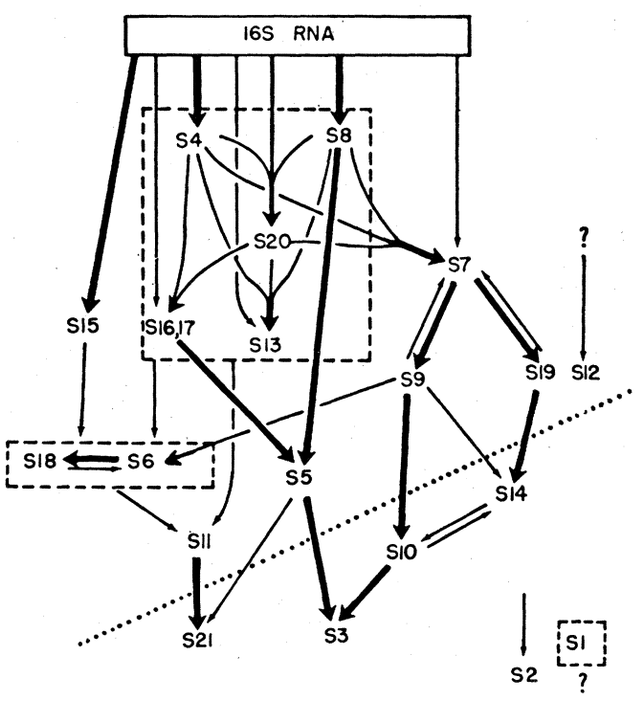


Fig. 1. The assembly map of 30*S* ribosomal proteins. The assembly map is based on the work of Mizushima and Nomura (*21*), slight modifications having been made as a result of subsequent work (*19, 42, 44*). The nomenclature of Wittmann *et al.* (*12*) is used instead of the previous nomenclature used in my laboratory. Arrows between proteins indicate the facilitatory effect on binding of one protein on another; a thick arrow indicates a major facilitatory effect. The map may be used to indicate the following relationships. The thick arrow from 16*S* RNA to S4 indicates that S4(P4a) binds directly to 16*S* RNA in the absence of other proteins. The thin arrow from 16*S* RNA to S7 indicates that S7(P5) binds weakly to 16*S* RNA in the absence of other ribosomal proteins. Thin arrows pointing toward S7 from S4(P4a), S8(P4b), S20(P14), S9(P8), and S19(P13) indicate that the latter proteins all help the binding of S7(P5) to RNA. The thick arrow from S7 to S19 indicates that in the absence of S7(P5), S19(P13) fails to bind the 16*S* rRNA containing complexes even in the presence of all other proteins under the standard reconstitution conditions. The arrow to S11(P7) from large box with dashed outline indicates that S11(P7) binding depends on some of the proteins enclosed in the box; it is not known exactly which proteins. The binding of S2(P2) and S12(P10) takes place at a later stage in the assembly sequence, but the exact position of these proteins in the map is not known (see *21*). S1(P1) does not bind under the conditions used. S16 and S17 were previously studied as a mixture, S17(P9b), but not S16(P9a), binds directly to 16*S* RNA (*47, 88*). The specificity of this binding has not been proved, and some investigators have not observed the direct binding of S13 to 16*S* RNA (*47*). Proteins above the dotted line are those either required for the formation of RI* particles or found in the isolated 21*S* RI particles (see text).

dyes should allow sensitive detection of conformational changes in ribosomal structure during assembly and during the various steps in protein synthesis. The resolution provided by this technique should far exceed that provided by any technique now in use. It is thus evident that many different approaches can soon be expected to yield much information on the three-dimensional structure of ribosomes.

Total Reconstitution of 50S Ribosomal Subunits

Although we encountered great difficulty in reconstituting *E. coli* 50*S* subunits in vitro, we recently succeeded in reconstituting 50*S* subunits obtained from the thermophilic organism, *B. stearothermophilus* (*32*).

In view of the results obtained with 30*S* reconstitution, it seemed reasonable to suppose that the difficulty with 50*S* reconstitution might reflect the greater complexity of the assembly reaction, as well as higher kinetic energy barriers which might be overcome only by longer incubation at even higher temperatures. The ribosomal components of *E. coli* 50*S* subunits or partially assembled intermediate particles might be too unstable to tolerate higher incubation temperatures. We found that reconstitution of 50*S* subunits in the *B. stearothermophilus* system, although possible, proceeds much more slowly than reconstitution of 30*S* subunits even at the optimum temperature (60°C) (*32*).

Initially, we used RNA and protein fractions obtained by dissociation of 50*S* subunits with 2*M* lithium chloride in 4*M* urea. The RNA fraction was shown to contain one protein still tightly bound to 23*S* rRNA. This protein, called L3, was not present in the 50*S* protein fraction obtained by this method. The protein L3 can be removed from 23*S* rRNA by treatment with acetic acid (66 percent) or with magnesium acetate at *p*H 2. We have subsequently demonstrated that functional 50*S* subunits can be reconstituted from a mixture of protein-free RNA with the 50*S* protein fraction plus the extracted protein L3 (*56*). The presence of L3 is essential for the reconstitution.

Studies on the effects of certain mutations on the assembly in vivo of *E. coli* ribosomes suggested that 30*S* subunits (or their precursors or components) play some crucial role in the assembly of 50*S* subunits (*57*). In earlier studies, the addition of 30*S* ribosomal subunits to the 50*S* reconstitution system occasionally stimulated the reconstitution of 50*S* subunits in vitro (*32*) but this stimulatory effect was not reproducible. Furthermore, when 50*S* and 30*S* subunits are reconstituted simultaneously from RNA and protein components derived from 70*S* ribosomes, the activity of the reconstituted 50*S* subunits is not significantly different from the activity obtained when the 50*S* subunits are reconstituted according to the standard procedure (*58*). Thus, the reconstitution of 50*S* subunits in vitro under the conditions described does not require the presence of 30*S* subunits (or their components). The role of 30*S* subunits in the assembly of 50*S* subunits in vivo remains unknown.

Studies of the 50*S* ribosome assembly system have shown that the presence of 5*S* RNA is required for reconstitution and have provided some information on the function of 5*S* RNA (*30, 31*). We have also demonstrated the role of methylation of 23*S* rRNA in the resistance of *S. aureus* to lincomycin (and to erythromycin and other related antibiotics) using a "hybrid reconstitution" system. By using dimethyladenine-containing 23*S* rRNA from resistant *S. aureus* strains, and other components (proteins and 5*S* RNA) from *B. stearothermophilus*, we obtained hybrid 50*S* subunits which were resistant to lincomycin; by replacing this 23*S* rRNA with 23*S* rRNA (lacking dimethyladenine) from a sensitive strain, we obtained particles that were sensitive to the drug (*41*).

To analyze the functions of 50*S* proteins, partial reconstitution systems obtained from *E. coli* have also been used. In earlier studies several 50*S* proteins were removed by high concentrations of CsCl as in the case of 30*S* subunits mentioned above, and functional analyses were done on these proteins (*59*). In pursuing this kind of approach, Nakamoto and his co-workers recently found that ethanol treatment removes a protein (or proteins) from 50*S* subunits and that this protein is essential for ribosomal functions involving both G and T factors, but is not required for the nonenzymatic binding of aminoacyl-tRNA or for the peptidyl transferase reaction (*60*). Similar results with what are presumably the same 50*S* proteins were obtained by other workers (*61*).

Several experiments have been done on RNA-protein interactions among components derived from the 50*S* subunits of *E. coli* under conditions which are defined to be optimum for 30*S* ribosomal reconstitution. For example, under such conditions 8 out of 34 50*S* proteins became bound to 23*S* RNA (*62*). Binding of 5*S* RNA to 23*S* RNA required the presence of only a few 50*S* proteins (*63*). However, because we lack a system for reconstitution of active *E. coli* 50*S* subunits, we cannot be sure whether the interactions studied are really pertinent to the structure of biologically active 50*S* ribosomal subunits.

There is a recent report that functionally active *E. coli* 50*S* subunits can be reconstituted from a mixture of 5*S* RNA, 23*S* RNA, and 50*S* proteins (*64*). We have not yet been able to reproduce these results and therefore cannot ascertain the importance of such a system in studies of *E. coli* 50*S* subunits.

Other Possible Ribosomal Functions

Since ribosomes comprise as much as a quarter of the total mass (dry weight) of a bacterial cell, and since they have a complex structure consisting of as many as 50 to 60 macromolecules, it is not unreasonable to suppose that ribosomes are engaged not only in protein synthesis, but also in other, unknown functions in vivo. Such multifunctional properties could be advantageous for coordination of several biosynthetic reactions. We should perhaps be willing to entertain this possibility.

An enzyme that participates in phospholipid biosynthesis in *E. coli*, phosphatidyl serine synthetase, is known to be associated with ribosomes and cannot be removed even after washing with buffers of high ionic strength (*65*). The significance of this association in phospholipid metabolism is unknown, but the possibility that it plays a role in coordinating lipid synthesis with protein synthesis has been suggested (*65*).

It has also been suggested (*66*) that ribosomes are involved in the regulation of rRNA synthesis by their direct participation in the metabolism of guanosine tetra- and pentaphosphates. This hypothesis is supported by the recent experiments of Haseltine *et al.* (*67*) who have shown that guanosine tetra- (and penta-) phosphates can be synthesized in vitro on the ribosome using guanosine diphosphate (and guanosine triphosphate) and ATP as substrates. Further studies on this subject would undoubtedly clarify the role of ribosomes in the regulation of rRNA synthesis.

Assembly of Ribosomes in vivo

The reconstitution of bacterial ribosomes in vitro has suggested the ordered sequence by which the 30*S* subunit is assembled from 16*S* RNA and its component proteins. It is likely that there is a similar ordered sequence of events in the assembly of 50*S* subunits in vitro (*19, 58*). That the sequential assembly of ribosomes also takes place in vivo is suggested by kinetic studies in which intermediate particles produced during ribosome synthesis can be detected in vivo by isotope labeling for limited periods (*68*). The assembly sequence has also been confirmed by the isolation of mutants which accumulate ribonucleoprotein precursors because of defects in assembly (*44, 57, 69*). In several instances, such ribonucleoprotein precursors were isolated and their protein compositions were analyzed (*44, 45, 70*). In the case of 30*S* precursors, protein compositions were compared with the map constructed for ribosome assembly in vitro. The 21*S* particles accumulated by some cold-sensitive mutants have protein compositions very similar to that of 21*S* RI particles isolated from reconstitution mixtures in vitro (*44*) (Table 1). On this basis, it has been concluded that the order of addition of proteins during 30*S* assembly in vivo is similar, if not identical, to the order of addition of proteins during the reconstitution of 30*S* subunits in vitro (*44*).

Although studies of reconstitution in vitro have provided much useful information on the assembly process of ribosomal particles, it is evident that the assembly processes in vivo and in vitro are not identical. One clear difference is the use of precursor 16*S* RNA and perhaps unmodified proteins in vivo. The RNA in the ribonucleoprotein precursor particles is slightly larger than mature 16*S* RNA, as determined by electrophoretic mobility in polyacrylamide gels (*71*) and by studies of the nucleotide sequence (*72*). The RNA is also submethylated or not methylated at all (*72*). During assembly of 30*S* subunits in vivo, some specific cleavages at both ends of the precursor 16*S* RNA must take place in addition to the methylation. Investigators are now attempting to identify and characterize these reactions in vitro (*73*). One reaction already identified is the formation of dimethyladenine. As mentioned before, kasugamycin-resistant mutants of *E. coli* lack methyl groups that are present in the dimethyladenine residues in the 16*S* RNA of wild-type kasugamycin-sensitive strains (*39*). An RNA methylase which has been isolated from the kasugamycin-sensitive strains is responsible for this methylation reaction and is absent from resistant mutants (*39*).

Some ribosomal proteins are known to have NH_2-terminal amino acids in which the NH_2 group of the amino acid is blocked (for example, *N*-acetylserine) or rare amino acids such as *N*-methyllysine (*74*). Thus some modifications of ribosomal proteins must occur after translation. During the head assembly of phage T4, some specific protein cleavages take place (*75*). Whether similar specific protein cleavages take place during ribosome assembly has not been studied.

Regulation of Ribosome Biosynthesis

The analysis of mutants that show defects in ribosome assembly (*69*) will probably provide much information on the detailed sequence of biochemical reactions and on the factors, ribosomal or nonribosomal, which control ribosome assembly. Useful information concerning intermediate particles and the possible "coupling" of 30*S* and 50*S* assembly, has already been obtained from such studies (*44, 57, 69, 76*). However, many problems still exist and have been discussed in detail in a recent review on the genetics of ribosomes (*4*). I will describe some of these problems very briefly.

Although the synthesis of rRNA has been studied extensively (*77*) the exact mechanism that regulates the synthesis is still unknown. Physiological studies (*78*) as well as electron microscopic studies (*79*) strongly suggest that the genes for 16*S*, 23*S*, and 5*S* rRNA's are in one single operon and are transcribed as a unit. This probably ensures the coordinated synthesis of 5*S*, 16*S*, and 23*S* rRNA. Such coordination would be necessary to make equal numbers of 30*S* and 50*S* ribosomal subunits in a growing cell. A simple and ingenious theory involving a protein factor known as ψ and guanosine tetra- (and penta-) phosphate has also been proposed (*80*) to explain the regulation of rRNA gene expression. Although the reported specific stimulation of rRNA gene transcription by the ψ factor is still controversial (*81*), there is a strong correlation between guanosine tetra- (and penta-) phosphate formation and the suppression of rRNA synthesis in vivo (*82*).

A formal genetic analysis of rRNA genes has never been made. Not only the genetic structure of a single rRNA gene operon and its regulation, but also the significance of the redundancy of such rRNA genes in a bacterial cell could well be studied by genetic approaches. This would be a challenging problem for bacterial geneticists.

Little information is available on the regulation of synthesis of ribosomal proteins (*4, 83*). The coordinated expression and the regulation of several nonribosomal bacterial genes have been studied by a variety of genetic approaches. One of the major obstacles to the use of such genetic approaches in studies of ribosomal protein genes is that many mutations of these genes are lethal, healthy ribosomes being essential for cell growth. To overcome this obstacle, studies have been initiated in our laboratory on ribosomal gene organization. For these studies we are using partial diploid *E. coli* strains which are heterozygous for the *str-spc* region on the chromosome. It is known that many genes coding for ribosomal proteins occur at the *str-spc* region (*4, 84*). Recent investigations of drug-resistant mutations induced by mu phage in such diploid strains have shown that a single mutational event caused by the insertion of mu phage affects the expression of more than one gene (*85*). It appears that many genes coding for ribosomal proteins which occur near the locus of *str* gene are transcribed as a single unit (ribosomal protein operon). Further studies of such diploid systems should provide useful information on the initiation site (promoter) of the ribosomal protein operon and the regulatory mechanisms controlling the expression of these genes. The transcriptional gene products, that is, the mRNA's for ribosomal proteins, have never been isolated. Even if we accept the existence of a large ribosomal protein operon, as deduced from the mu insertion experiments, this does not mean that all the proteins are synthesized on a single polycistronic mRNA. It is possible that the initial transcriptional product undergoes posttranscriptional cleavages and modifications.

Finally, there is the problem of how the regulatory processes for the expression of rRNA genes and ribosomal protein genes are coordinated. That such coordination occurs in vivo seems highly likely. Possible mechanisms for coordination have been discussed previously (*4, 83*) but there is no experimental proof to support any of these mechanisms exist.

Conclusions

I have not mentioned the remarkable progress made mainly by Fellner and his co-workers (*86*) in the elucidation of the primary structure of rRNA's and by Wittmann and his co-workers (*87*) in determining the structure of several ribosomal proteins. Such knowledge of primary structures is certainly the basis of complete understanding of the structure of the ribosome. With the current progress in technology, complete elucidation of the primary structure of all the ribosomal components is probably a matter of time. As indicated in this article, a rough approximation of the three-dimensional structure of ribosomes is likely to emerge soon. Although not mentioned in this article, studies of ribosomes from higher organisms are also progressing. We must, therefore, consider what further studies should be conducted and what kinds of questions we would like to solve.

Some groups of investigators aim to elucidate the complete three-dimensional structure of ribosomes and to find out how these complex cell organelles function; they hope to determine the conformational changes of many of the component molecules within the ribosome structure in response to external macromolecules and cofactors engaged in protein synthesis. Such knowledge will also be important in enabling us to understand the regulation of translation of genetic messages. Other groups of investigators aim to elucidate the complex series of events which originate in the transcription of the more than 60 genes and culminate in the formation of the specific structure of the organelle. Complete reproduction in vitro of all the assembly events that occur in vivo should not be difficult to achieve in principle. It should then become possible to study in vitro any factor regulating the biogenesis of the organelle. Although we do not know whether such studies would reveal any new fundamental principle that governs the complex circuits of interconnected macromolecular interactions, the achievement of such a complete in vitro system would represent a necessary step in the comprehensive understanding of biogenesis of organelles, and eventually, of the more complex behavior and genesis of cells (*89*).

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→ M. Nomura and F. Engbaek, *Proc. Nat. Acad. Sci. U.S.A.* **69**, 1526 (1972). Phage mu is known to cause mutation in many bacterial genes by the direct integration into the gene [→ A. L. Taylor, *ibid.* **50**, 1043 (1963); W. Boram and J. Abelson, *J. Mol. Biol.* **62**, 171 (1971); A. J. Bukhari and D. Zipser, *Nature New Biol.* **236**, 240 (1972)]. The insertion of the mu prophage DNA into any bacterial operons should interfere with the transcription of the cistrons distal to the initiation site (the "promotor"), but not the cistrons proximal to the promotor. This principle has been confirmed experimentally [see E. Jordon, H. Saedler, P. Starlinger, *Mol. Gen. Genet.* **102**, 353 (1968); A. Touissaint, *ibid.* **106**, 89 (1969)] and was used in the experiment of Nomura and Engbaek to test if four drug resistant genes at the *str-spc* region are in the same operon.
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89. This manuscript was written in April 1972. Several papers which are pertinent to the discussion but were published thereafter may not be mentioned. Supported by grant GM-15422 from the National Institutes of Health and by grant GB-31086X from the National Science Foundation. This is Paper No. 1553 of the Laboratory of Genetics.

Ambiguities in the Use of Unit Names

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Experimental determinations of physical constants are usually reported in the literature in accepted units, such as SI (*1*). The actual measurements are *not* made in terms of the reported units, but in terms of the units maintained by a standards laboratory. This lack of precise identification of the results leads to ambiguities in comparing the data from different countries, and in adjusting constants to make a most consistent set.

For example, when a determination of the gyromagnetic ratio of the proton is reported, there is not only an experimental uncertainty associated with the calibration of the equipment, but usually also a semantic uncertainty associated with the meaning of the names of the units given. For example, if the gyromagnetic ratio is reported as 42.5764 MHz/T, what is really meant by "tesla"?

The ambiguity surfaces when an attempt is made to compare results given before and after the 1969 adjustment to the volt as maintained by national laboratories. The tesla is proportional to the volt by

$$T = V \cdot s/m^2$$

but also inversely proportional by

$$T = kg \cdot \Omega/s^2V$$

The volt as maintained in the United States was decreased by 8.4 ppm (1 January 1969), with no change in the ohm; should previously published values of the gyromagnetic ratio be increased or decreased for comparison with later measurements?

Theoretical Units and Actual Measurement Units

The SI base units of mechanics and electromagnetism are the kg, m, s, and A. In actual measurements, however, a laboratory uses units disseminated by national laboratories via a calibration process. For the scope of this discussion, let us assume that the "local" kg, m, and s are essentially perfect, and that they are used for measuring mass, length, and time. Measurements of force, and of electric quantities, are made by comparison with imperfect standards. Let us denote the local units maintained by these imperfect standards by $\hat{N}$, $\hat{V}$, $\hat{\Omega}$, $\hat{F}$; these are approximations to the true newton, volt, ohm, and farad. The three electrical units are established by calibrations of cells, resistors, and capacitors, and are algebraically and physically independent. The laboratory newton is usually real-

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