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Reconstitution of 50S Ribosomal Subunits from Protein-Free Ribonucleic Acid†

S. Fahnestock,‡ V. Erdmann,§ and M. Nomura*

ABSTRACT: Previous reconstitution studies on the 50S ribosomal subunit of *Bacillus stearothermophilus* used as starting material a complex of 23S rRNA with a 50S ribosomal protein, L3. This protein can be removed from 23S RNA at pH 2.0 in the presence of 4 M urea and 0.5 M Mg^{2+}, and active 50S subunits can then be reconstituted from the resulting protein-free 23S RNA. Thus, the 50S ribosomal subunit can be completely self-assembling. Protein L3 is not important for the physical assembly of the 50S subunit, but is required for the reconstitution of active particles. Particles reconstituted without L3 are deficient in several different ribosomal functions.

The large ribosomal subunit from *Bacillus stearothermophilus* can be reconstituted *in vitro* from dissociated RNA and protein fractions (Nomura and Erdmann, 1970). In previous studies we have made use of a reconstitution system utilizing RNA and protein fractions obtained by dissociation with 4 M urea–2 M LiCl (Nomura and Erdmann, 1970; Erdmann *et al.*, 1971a; Fahnestock and Nomura, 1972). As we have previously described, 23S rRNA prepared in this way retains several proteins still bound to it (Nomura and Erdmann, 1970; Erdmann *et al.*, 1971b). One of these proteins, which we have designated L3, is present only in small amounts in the urea–LiCl protein fraction, being found mostly in the RNA fraction; several other proteins which are found in the RNA fraction are present in larger amounts in the protein fraction.

Since RNA obtained by the urea–LiCl precipitation method ("urea–LiCl RNA" in this paper) retains bound protein, the above system cannot strictly be considered a complete reconstitution. It is possible that the 23S RNA–L3 complex retains some structural features of the 50S subunit which might not be retained by the free RNA and protein. In order to determine whether the 50S ribosomal subunit can be entirely self-assembling *in vitro* or whether perhaps the L3–RNA complex retains some important assembly information which might result from some extraribosomal influence *in vivo*, it is necessary to reconstitute the 50S subunit from protein-free RNA. Here we describe the total reconstitution of the *B. stearothermophilus* 50S subunit, together with some properties of the protein L3.

Materials and Methods

Ribosomes were obtained from *B. stearothermophilus* and washed through sucrose and 0.5 M NH_4Cl as described previously (Fahnestock and Nomura, 1972). Purified 50S subunits were obtained as described (Erdmann *et al.*, 1971a).

A mixture of (undissociated) 50S and 30S ribosomal subunits was treated with 4 M urea–2 M LiCl for 36–48 hr at 0° to obtain RNA and protein fractions (Nomura and Erdmann, 1970). The RNA fraction was freed of remaining protein by treatment with 4 M urea–0.5 M $MgAc_2$ (pH 2.0). The RNA pellet obtained from the urea–LiCl treatment, after washing with 4 M urea–2 M LiCl, was redissolved in 6 M urea to a concentration of 10–20 mg/ml. To this solution was added one-third volume 2 M $MgAc_2$ which had been adjusted to pH 2.0 with HCl. After 1 hr at 0°, the precipitated RNA was collected by centrifugation, suspended in a buffer (TMA-II) containing 10 mM Tris-HCl (pH 7.4), 0.3 mM $MgCl_2$, 30 mM NH_4Cl, and 6 mM 2-mercaptoethanol, and dialyzed until dissolved (4 hr) against the same buffer. The supernatant containing protein L3 (crude L3) was dialyzed 6 hr against a buffer (TRI) composed of 30 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$, 1 M KCl, and 6 mM 2-mercaptoethanol.

Purification of L3. Protein L3, identified by its mobility on two-dimensional polyacrylamide gel electrophoresis (see Figure 5), was purified from the pH 2 urea–Mg supernatant by chromatography on carboxymethylcellulose. Protein from 100 mg of 70S ribosomes was applied to a 3-ml column of Whatman CM52 equilibrated with buffer A, composed of 16 ml of pyridine, 9.6 ml of formic acid (88%), and 1 ml of 2-

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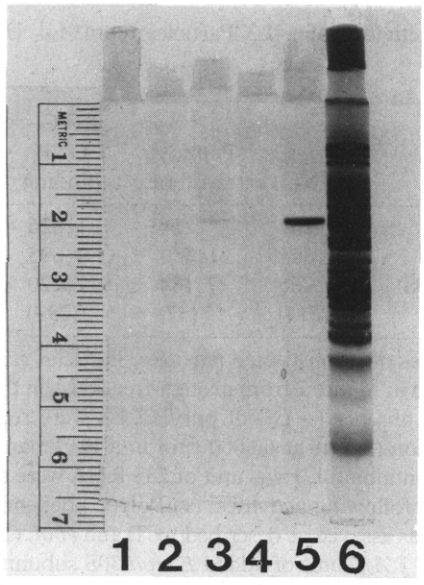

FIGURE 1: Polyacrylamide gel electrophoretic protein analysis of RNA fractions and L3. Sample preparation and electrophoresis were carried out as described by Traub *et al.* (1971) in 10% polyacrylamide gels at pH 4.5. (1) Blank gel; (2) 11 A_{260} units of RNA, derived from a mixture of 50S and 30S subunits, preparation A; (3) 22 A_{260} units of RNA, preparation A; (4) 18 A_{260} units of RNA, preparation B; (5) 10 A_{260} equiv units of purified L3; (6) 7 A_{260} units of 50S subunits. RNA preparation A was used for all experiments except that shown in Table I, for which preparation B was used, and that shown in Figure 4, for which a third preparation, not shown, was used. All the preparations were prepared by the same method.

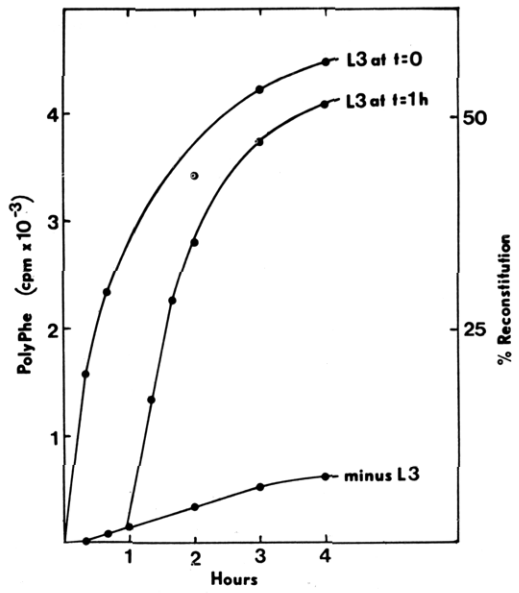

FIGURE 2: Reconstitution of active 50S subunits from protein-free RNA. Each 0.9-ml reconstitution mixture contained 0.03 M Tris-HCl (pH 7.4) (23°), 0.02 M $MgCl_2$, 0.33 M KCl, 0.006 M mercaptoethanol, 9 A_{260} units of 23S RNA (12 A_{260} units of RNA, derived from a mixture of 50S and 30S subunits), and 11 50S equiv units of urea–LiCl protein fraction (defined as the amount of protein obtained by this procedure from 11 A_{260} units of 50S subunits) derived from mixed 30S and 50S subunits. Crude L3 (2 50S equiv units/A_{260} unit of 23S RNA) was added at $t = 0$ or $t = 1$ hr. Samples were incubated at 60° from $t = 0$, and aliquots (0.1 ml) were removed for assay at various times. Poly(U)-dependent poly(phenylalanine) synthesis was assayed as described by Traub *et al.* (1971) but with the following modification. A 0.1-ml aliquot of the reconstitution mixture was added to 0.1-ml assay mixture containing all of the ingredients described by Traub *et al.* (1971) plus 1 A_{260} unit of *E. coli* Q13 30S subunits. Per cent reconstitution was determined by comparison with the activity of undissociated ribosomes assayed in the same way.

mercaptoethanol per l. of 6 M urea. The column was eluted with a 60-ml linear gradient from buffer A to buffer B, composed of 48 ml of pyridine, 19.2 ml of formic acid, and 1 ml of 2-mercaptoethanol per l. of 6 M urea. This column procedure was adapted from the system described by Hindennach *et al.* (1971). Fractions were analyzed by polyacrylamide gel electrophoresis (Traub *et al.*, 1971) and the appropriate fractions were pooled and concentrated in dialysis membrane by packing in solid sucrose, then dialyzed against TRI.

The reconstitution conditions were as described previously (Nomura and Erdmann, 1970) and in Figure 2. All RNA and protein preparations were derived from a mixture of 30S and 50S subunits, except that used for the experiment shown in Figure 4, which was derived from purified 50S subunits.

Results

Reconstitution of 50S Ribosomal Subunits from Protein-Free RNA. Treatment of urea–LiCl RNA fraction derived from 70S or 50S ribosomes with 0.5 M $MgAc_2$ at pH 2.0 in 4 M urea removes residual protein and results in a preparation which is essentially free of protein. Protein analysis of two different preparations of RNA prepared as described in the Methods section is shown in Figure 1. It was estimated that preparation A is at least 95% free of L3, and preparation B is at least 98% free of L3. No other bands are detectable.

Figure 2 shows the reconstitution of 50S activity from protein-free RNA. In this figure "L3" refers to the crude pH 2 urea–Mg^{2+} supernatant fraction. The rate of reconstitution and amount of activity obtained in the presence of L3 are comparable to the values obtained in our previous reconstitutions, using urea–LiCl RNA. The reconstitution is strongly dependent on the addition of crude L3 fraction. We conclude that the 50S subunit can be reconstituted from protein-free RNA and a mixture of proteins; the protein–RNA complexes which are preserved in urea–LiCl RNA can be dissociated, and reformed during reconstitution in this system.

Role of Protein L3. Figure 2 also indicates the importance of the proteins which are present in the urea–LiCl RNA. There are four detectable protein bands in this fraction. These have been separated by chromatography on carboxymethylcellulose. Other experiments showed that only protein L3 is capable of restoring reconstitution activity. The other proteins, while slightly stimulatory in the presence of L3, do not restore activity in the absence of L3.

Protein L3, purified in this way, consists of a single band after electrophoresis in polyacrylamide gels in urea at pH 4.5 (Figure 1, tube 5) and in urea–sodium dodecyl sulfate. Its molecular weight, determined by sodium dodecyl sulfate gel electrophoresis (Weber and Osborn, 1969) using as standards bovine serum albumin, ovalbumin, carboxypeptidase B, trypsin, and lysozyme, is 35,000 ± 3000.

Figure 3 shows the dependence of reconstituted 50S activity on the amount of added pure L3. This data suggests the requirement for 1 mol of L3/mol of 23S RNA, but cannot rule out the (less likely) possibility that 2 mol are required. An important uncertainty in these data arises from the fact that the concentration of L3 was determined by the method of Lowry *et al.* (1951) using as a standard bovine serum albumin. Thus, the scale of the abscissa is in error by the ratio of the color yields from the two proteins.

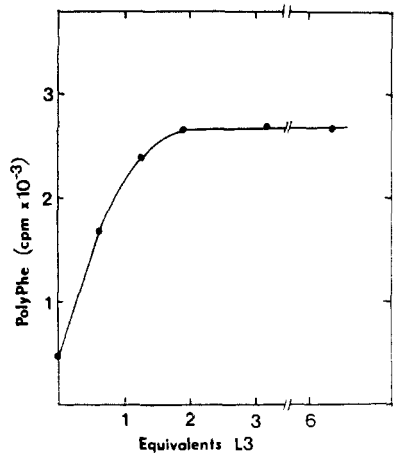

FIGURE 3: Dependence of reconstituted 50S activity on protein L3. Reconstitution was carried out as described in Figure 2 in the presence of various amounts of purified protein L3 (expressed as mol of L3/mol of 23S RNA). The concentration of L3 was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. After 2 hr at 60°, 0.1-ml aliquots containing 1 A_{260} unit of 23S RNA were assayed directly for poly(U)-dependent $(Phe)_n$ synthesis as in Figure 2.

When L3 is omitted from the reconstitution system the resulting activity in several assays is greatly reduced (Table I). The activity of particles reconstituted in the absence of L3 ([−L3] particles) is 10% of the activity of particles reconstituted with L3 ([+L3] particles) in poly(U)-dependent $(Phe)_n$ synthesis. Peptidyltransferase activity, measured by the formation of formylmethionylpuromycin from fMet-tRNA and puromycin in the presence of 33% methanol, is also reduced, as is the ability to form 70S couples, measured by protection of Phe-tRNA bound to 30S ribosomes from ribonuclease digestion. The EF-G-dependent binding of GTP is also reduced, though perhaps less than the other activities.

Sucrose gradient sedimentation analysis of [+L3] and [−L3] particles is shown in Figure 4. [+L3] particles, like

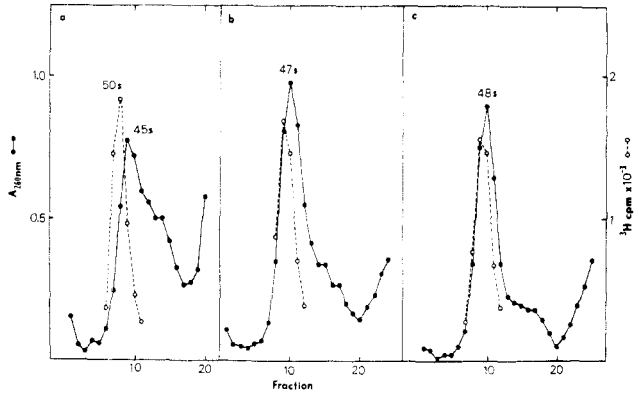

FIGURE 4: Sucrose density gradient analysis of reconstituted particles. RNA and protein fractions derived from purified 50S subunits were used for this experiment. Particles were reconstituted as in Table I from protein-free 23S RNA and urea–LiCl protein in the presence (b) and absence (a) of the pH 2 urea–Mg^{2+} supernatant (crude L3). Particles were also reconstituted from the L3-containing urea–LiCl 23S RNA (c). Aliquots were analyzed by sedimentation through sucrose gradients (5–20%) containing TMA-II buffer along with a marker, [^{3}H]uracil-labeled 50S subunits, for 90 min at 50,000 rpm in a Beckman SW50 rotor. S values are approximate, and were obtained by comparison with the marker.

TABLE I: Activities of −L3 Particles (cpm/A_{260} Unit of 23S RNA).[a]

	Poly(U)	Peptidyl-transferase	EF-G–GTP Binding	Phe-tRNA Binding
−L3	257	214	1,826	103
+L3	2,565	1,480	4,695	918
Native 70S	10,265	7,184	12,220	1,225
(Blank)	(214)	(57)	(297)	(351)

[a] Activities of reconstituted particles. Particles reconstituted as described in Figure 2 from protein-free RNA in the presence (+L3) and absence (−L3) of purified L3 were recovered by centrifugation (2.5 hr at 60,000 rpm in a Beckman 65 rotor). Aliquots containing 1.0 A_{260} unit of 23S RNA were assayed for each of the following activities. (1) Poly(U)-dependent $(Phe)_n$ synthesis was assayed as described by Traub *et al.* (1971) in the presence of 1 A_{260} unit of added *E. coli* 30S subunits. (2) Peptidyltransferase activity was determined by the formation of fMet-puromycin from fMet-tRNA and puromycin in the presence of methanol as described previously (Erdmann *et al.*, 1971a). (3) G factor dependent binding of [^{3}H]GTP was assayed by the method of Bodley *et al.* (1969) as described previously (Erdmann *et al.*, 1971a). (4) Phe-tRNA binding was assayed by the method of Pestka (1968) as described previously (Erdmann *et al.*, 1971a) in the presence of 0.5 A_{260} unit of added *E. coli* 30S subunits. This assay depends on the protection of tRNA from RNase afforded by the formation of a 70S couple. In each assay an identical sample of the same 70S ribosomes used to prepare the RNA and protein components was run for comparison along with a blank, containing no 50S subunits. The blank value (in parentheses) was subtracted to obtain the data shown.

particles reconstituted from urea–LiCl RNA, sediment at 47–48 S, somewhat more slowly than marker native 50S subunits. [−L3] particles are somewhat more heterogeneous, with a peak at about 45 S.

The protein composition of the 45S material was analyzed using the two-dimensional polyacrylamide gel electrophoresis technique of Kaltschmidt and Wittmann (1970). As shown in Figure 5, the only qualitative difference between the patterns obtained from sucrose gradient purified [−L3] particles (45S material) and [+L3] particles is in the L3 spot itself. Aside from L3, the patterns are indistinguishable.

The sedimentation pattern shown in Figure 4a and the protein composition in Figure 5 represent the product of a 60-min reconstitution in the absence of L3. Most of the 23S RNA has been incorporated into 45S material. As shown in Figure 1, if L3 is added after 1 hr during which 45S particles are formed, the final yield of active ribosomes is changed only slightly, and no time lag is observed in the kinetics of formation of active particles. Thus, L3 can be incorporated to form an active 50S subunit after all the other 50S proteins are bound to RNA.

Discussion

These experiments demonstrate the complete self-assembling ability of the 50S ribosomal subunit *in vitro*. Whether the

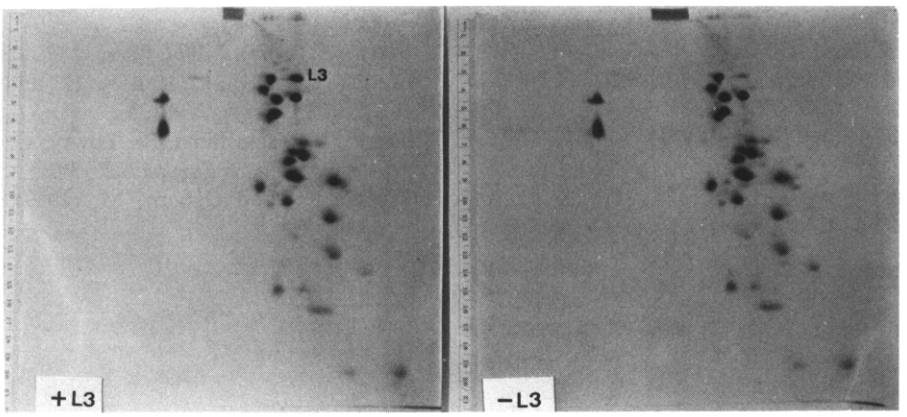

FIGURE 5: Protein composition of reconstituted particles. Particles were reconstituted as in Table I with and without pure L3. The fastest running (23S RNA-containing) component was purified by centrifugation through a 10–30% sucrose gradient containing TMA-II buffer for 15 hr at 23,000 rpm in a Beckman SW27 rotor. Protein samples were prepared by RNase digestion (Traub *et al.*, 1971) and analyzed by two-dimensional polyacrylamide gel electrophoresis (Kaltschmidt and Wittmann, 1970) using the pH 8.7 first-dimension system.

50S subunit is self-assembling *in vivo* remains unknown. Clearly the *in vitro* system is inefficient compared to the *in vivo* process, and may differ from it in some important respects. There is some evidence that the assembly of 50S subunits *in vivo* in *Escherichia coli* depends upon the simultaneous assembly of 30S subunits (Nashimoto and Nomura, 1970; Kreider and Brownstein, 1971). This dependence is not reproducible in the *Bacillus* system *in vitro*, either in the present complete reconstitution system or in the system using urea–LiCl 23S RNA. The addition of 30S subunits or the simultaneous reconstitution of 30S subunits from urea–LiCl RNA and proteins has no effect on the reconstitution of 50S subunits in either system (Fahnestock *et al.*, 1973).

Protein L3 is necessary for the assembly of active 50S subunits, but is not required for the binding of any other protein to the particle. In its absence a particle is assembled which contains all other 50S proteins but is inactive. Therefore, protein L3, while apparently not important in the physical assembly of ribosomes, has an important function in the assembled ribosome. All activities which we have measured, including peptidyltransferase activity, ability to bind G factor and GTP, and ability to interact with 30S subunit–Phe-tRNA–poly(U) complexes, are dependent on the presence of the single protein L3. This indicates a certain interdependency among the various independently measurable functions of the 50S subunit. One possibility is that protein L3 is located in a critical region of the ribosome at or near which all of these functions take place. Equally likely, however, is the possibility that the absence of L3 alters the overall structure of the 50S subunit, possibly affecting widely separated regions. If this is the case, then the fact that all other proteins can bind to such an altered particle implies that binding sites for individual ribosomal proteins are less dependent on the overall ribosome conformation than is any of the activities we have measured.

The functional properties of L3 are somewhat different from those of any of the *E. coli* 30S proteins. Many of the 30S proteins which, like L3, bind directly to (16S) RNA are, unlike L3, strongly required for the assembly of the subunit (Nomura *et al.*, 1969). Some of the direct binding 30S proteins (S15(P10b) and S20(P14) and possibly S13(P10a)) do resemble L3 in having little effect on the physical assembly of the subunit. However, these proteins are not entirely essential for the activity of reconstituted particles (Nomura *et al.* (1969), and W. Held and M. Nomura, unpublished data) in contrast to the functional importance of L3. For example, particles lacking either S15 or S20 are fully active in the binding of aminoacyl-tRNA in the presence of 0.02 M Mg^{2+} ions. Thus, the importance of L3 for a variety of different activities, its unimportance for subunit assembly and its ability to bind directly to rRNA are a combination of properties unique among ribosomal proteins which have been examined so far.

Stöffler *et al.* (1971) have shown that *B. stearothermophilus* protein L3 is immunologically related to one of two *E. coli* 50S proteins, L2 or L24. Both of these *E. coli* proteins bind directly to 23S RNA under the ionic conditions optimal for the 30S assembly and one, L2, has a mobility in two-dimensional gel electrophoresis (Hindennach *et al.*, 1971) and a molecular weight (Dzionara *et al.*, 1970) very similar to that of *B. stearothermophilus* L3. It is, therefore, possible that *B. stearothermophilus* L3 and *E. coli* L2 are analogous in terms of their structural and functional roles in their respective 50S subunits.

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Selective Stimulation of Nonhistone Chromatin Protein Synthesis in Lymphoid Cells by Phytohemagglutinin†

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ABSTRACT: The chromatin proteins synthesized by phytohemagglutinin-stimulated lymphocytes have been investigated using a double isotope labeling approach. Lymphocyte chromatin was dissociated in 0.4 M guanidine-HCl, 6 M urea, 0.1% β-mercaptoethanol, and 0.1 M sodium phosphate buffer at pH 7.0, which solubilized >90% of the total chromatin proteins. The histones and nonhistones were then separated from one another by ion exchange chromatography and analyzed by polyacrylamide gel electrophoresis. The nonhistone chromatin proteins accounted for less than 15% of the total protein in lymphocyte chromatin but they contained >70% of the [^{3}H]leucine incorporated into the chromatin protein during a 2-hr pulse. When lymphocytes were stimulated by phytohemagglutinin, there was a prompt increase in the synthesis of all the nonhistone proteins, but not of histones. Among the nonhistones, several specific proteins were preferentially synthesized during the activation of the cell by phytohemagglutinin.

The nonhistone, or acidic, chromatin proteins have been implicated in control of gene expression in eukaryotic organisms. This view is based primarily on demonstrations that nonhistone chromatin proteins show tissue specificity (Elgin and Bonner, 1970; Levy *et al.*, 1972), are present in increased amounts in metabolically active tissues (Dingman and Sporn, 1964; Bonner *et al.*, 1968) and euchromatin (Frenster, 1965) and can increase *in vitro* template activity of various preparations of chromatin for RNA polymerases (Gilmour and Paul, 1969; Wang, 1970). Specifically, nonhistone chromatin proteins have been suggested as a controlling element in the gene activation which occurs prior to mitosis in animal cells. For recent reviews see Stellwagen and Cole (1969a) and Stein and Baserga (1970).

Recently, we have developed a general method for the fractionation of chromatin (Levy *et al.*, 1972). Utilizing this method, nucleic acids, histones, and nonhistone proteins are separated from one another and quantitatively recovered from a single chromatin sample. We have now applied this methodology to extend our previous studies on the proteins of stimulated lymphocytes (Levy and Rosenberg, 1972a,b; Rosenberg and Levy, 1972). We have found that an increase in the rate of synthesis of nonhistone chromatin proteins is one of the earliest processes during the activation of the lymphoid cell by phytohemagglutinin. Moreover, among these proteins, certain specific nonhistones are preferentially stimulated.

Experimental Section

Lymphoid Cell Culture. The mesenteric lymph nodes of strain 2 guinea pigs (Animal Production Unit, National Institutes of Health) were removed, dissected free of fat and connective tissue, and gently teased apart with fine tipped forceps into tissue culture medium (Eagle's minimal essential medium lacking leucine but supplemented with penicillin (100 u/ml), streptomycin (100 μg/ml), glutamine (0.59 mg/ml), and nonessential amino acids). The larger particles were allowed to settle for 2 min and the lymphocytes in the supernatant were collected by centrifugation at 300*g* at room temperature, washed once, and diluted to a concentration of 10^7 cells/ml in leucine-free medium. Cell suspensions were cultured at 37° in a humidified atmosphere of 10% CO_2 in air. For considerations of geometry and gas equilibration, these incubations were conducted in 1-ml aliquots in round bottom 16 × 125 mm tubes.

PHA[1] Stimulation and Isotope Labeling. Phytohemagglutinin was dissolved at 100 μg/ml in a sterile solution of 0.14 M NaCl buffered at pH 7.2 with 0.007 M sodium phosphate and 0.1 ml was added to half the cultures. The other half of the cultures received the same volume of buffered saline alone. At various times thereafter, PHA and control cultures were

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[1] Abbreviation used is: PHA, phytohemagglutinin.