

# Endotoxicosis Induced by *Coxiella burnetii* Lipopolysaccharide Stimulates a Ribosomal Protein S6 Kinase: Some Properties of the Partially Purified Enzyme

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**Guinea pig endotoxicosis induced by lipopolysaccharide from *Coxiella burnetii* Nine Mile phase I stimulates phosphorylation of liver ribosomal protein S6, with a 50% increase at 12 h postinoculation. The responsible protein kinase (S6PK) has been partially purified from liver; its activity is independent of cyclic AMP and of Ca<sup>2+</sup> plus phosphatidyl serine or diacylglycerol. The preparation has an apparent optimum concentration of 20 mM Mg<sup>2+</sup>, while Ca<sup>2+</sup> and Mn<sup>2+</sup> are each inhibitory at 2 mM. The apparent K<sub>m</sub> for ATP is 30 μM with intact ribosomes. Because of the central role of phosphorylation in metabolic regulation and a purported role of phosphorylated S6 in protein synthesis, the lipopolysaccharide-induced stimulation of S6PK suggests a significant regulatory role of such enzymes in the pathobiochemistry of Q fever infection and endotoxicosis.**

Aspects of the pathobiochemistry of Q fever and of the endotoxicosis caused by the lipopolysaccharide (LPS) of the rickettsial agent have been reported elsewhere (1). Cortisol (30) and cyclic AMP (cAMP) (20) levels increase during Q fever. Stimulated synthesis of RNA (30); increased activities of RNA polymerases I, II, and III (22); and phosphorylation of nonhistone chromatin proteins (9) are correlated. Enhanced liver protein synthesis and phosphorylation of liver ribosomal protein S6 (14) are parallel. In vitro translation systems prepared from rabbit reticulocytes have greater activity with phosphorylated S6 guinea pig liver ribosomes (14), which suggests some relationship between protein synthesis and ribosomal protein phosphorylation. The multiple regulatory roles of phosphorylation in transcription and translation, the unresolved questions of protein synthesis regulation in Q fever or its endotoxicosis, and the associated question of phosphorylated S6 in protein synthesis (32) led to the present study.

## MATERIALS AND METHODS

**Animals and LPS.** Male Hartley strain guinea pigs, each weighing 250 to 300 g, were from Hilltop Lab Animals, Inc. (Scottsdale, Pa.). LPS was prepared from the rickettsial agent of Q fever, *Coxiella burnetii* Nine Mile phase I (12).

**Liver extracts.** Control animals (N) were injected intraperitoneally with 0.4 ml of buffer (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.2], 0.15 M NaCl). Experimental animals (L) were injected with 400 μg of LPS per 0.4 ml of buffer. Animal temperatures were recorded at this time and at sacrifice. Animals were sacrificed 12 h postinoculation and exsanguinated. Livers were rapidly excised, washed in ice-cold 0.15 M NaCl, blotted, and weighed. Subsequent steps were conducted at 4°C.

A 15% (wt/vol) liver homogenate was prepared in 10 mM Tris buffer (pH 7.6)–7 mM β-mercaptoethanol–1 mM sodium orthovanadate (buffer A) with a Tekmar homogenizer. Orthovanadate and β-glycerophosphate are active phosphatase inhibitors (5). The inhibitory activities of both were com-

pared under similar conditions; the results were similar (data not shown), and orthovanadate was used in further work. Insoluble material was removed by centrifuging at 10,000 × g for 15 min. A postribosomal supernatant was prepared by centrifugation at 25,000 rpm in a model SW41 Ti rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 1 h. Stepwise additions of ammonium sulfate were made, and the 20 to 45% fraction was stirred overnight. The precipitated protein was collected, suspended in buffer A, and desalted using Econo-Pac 10 DG columns (Bio-Rad Laboratories, Richmond, Calif.). The eluate was chromatographed on DEAE-Sephadex equilibrated with buffer A and eluted using stepwise-increased concentrations of NaCl (Fig. 1A). The fractions were assayed for protein kinase (PK) activity against intact control ribosomes. Active fractions were pooled and layered over linear gradients of 12 to 36% (wt/vol) glycerol in buffer A. After centrifugation at 160,000 × g for 41 h, 0.5-ml fractions were collected and assayed for PK activity (Fig. 1B). The apparent M<sub>r</sub> was measured by fast protein liquid chromatography gel filtration on a column (1 by 30 cm) with Superose 12 (Pharmacia, Inc., Piscataway, N.J.) at a rate of 0.4 ml/min, and 0.4-ml fractions were collected (Fig. 1C). Protein was measured by the method of Bradford (3).

**PK assay.** To a reaction mixture containing (final concentration) 10 mM HEPES (pH 7.2), 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.2 mM EDTA, 0.25 mM EGTA, and 100 μM [γ-<sup>32</sup>P]ATP (specific activity, 0.1 Ci/mmol) were added 10 μl of sample and either 50 μg of intact control ribosomes (31) or 20 μg of extracted ribosomal proteins in a final volume of 50 μl. After incubation at 30°C for the indicated times, the mixture was chilled in an ice-ethanol bath, and 25 μl was reacted with 25% (wt/vol) trichloroacetic acid. The precipitate was filtered through a model 934-AH glass fiber filter (Whatman, Inc., Clifton, N.J.), washed, dried, and counted by liquid scintillation spectrometry. PK activity for S6 was identified by one-dimensional (1D) and two-dimensional (2D) gel electrophoresis with autoradiography (14). To facilitate autoradiography of 2D gels, the specific activity of the [γ-<sup>32</sup>P]ATP was increased to 1.0 Ci/mmol in these reactions.

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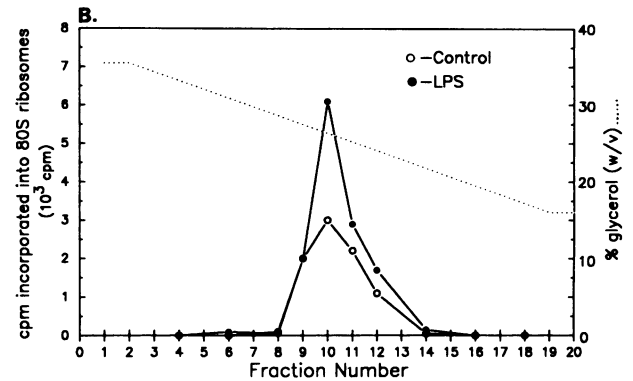
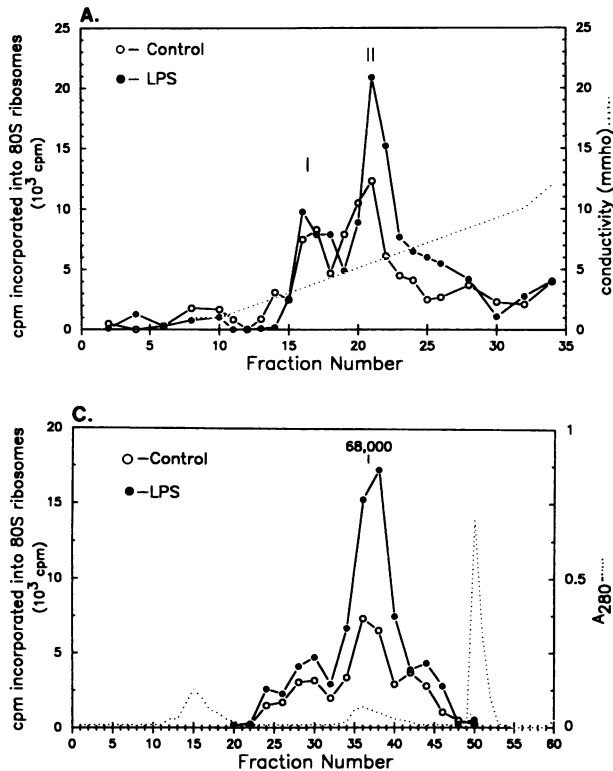


FIG. 1. Ribosomal PK purification. (A) DEAE chromatography of active PK from ammonium sulfate fractionation (see Materials and Methods); 2-ml fractions were collected for assay. (B) Active peak II fractions from panel A were applied to glycerol gradients and centrifuged (see Materials and Methods); 0.5-ml fractions were collected for assay. (C) Active fractions from panel B were further purified by Superose 12 gel filtration, and fractions for assay (see Materials and Methods) and gel electrophoresis (Fig. 5) were collected.  $M_r$  standards for gel filtration were ferritin, alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase, and cytochrome *c*.

Mild trypsin-mediated proteolysis was done as described by Perisic and Traugh (24).

**Reagents and polyacrylamide gel electrophoresis.** [ $\gamma$ - $^{32}$ P] ATP was synthesized with the Gamma-Prep A system (Promega Biotec, Madison, Wis.) and carrier-free [ $^{32}$ P]H<sub>3</sub>PO<sub>4</sub> (ICN Pharmaceuticals Inc., Irvine, Calif.) according to the instructions of the manufacturer.

Histones H1, H2A, H2B, and H3, mixed histones (type II-S), and dephosphorylated casein were from Sigma Chemical Co. (St. Louis, Mo.);  $\alpha$ S<sub>1</sub>-casein was a gift from E. Bingham, Department of Agriculture, Philadelphia, Pa. Ribosomes used in PK assays were prepared from control animal livers as previously described (19). Basic ribosomal proteins were extracted with acetic acid from samples of these ribosomes (27).

The effects of agonists or antagonists on enzyme activity were measured by using 0.2 mg of heat-stable inhibitor of cAMP-dependent PK per ml, 2  $\mu$ M cAMP, 2 mM Ca<sup>2+</sup>, 10  $\mu$ g of phosphatidyl serine or dioctanoylglycerol per ml, 300  $\mu$ g of heparin per ml, 10 mM *N*-ethylmaleimide, and 50  $\mu$ g of sphingosine per ml.

Because S6 phosphorylation qualitatively correlated with filter paper counts, the latter was used as a convenient index of S6 kinase activity against  $\alpha$ S<sub>1</sub>-casein, dephosphorylated casein, phosvitin, and histones. A 1D sodium dodecyl phosphate-gel electrophoresis was performed by the method of Laemmli (16), and 2D gel electrophoresis was performed by the method of Madjar et al. (18) as modified by Hickey et al. (14). Gels were autoradiographed as described by Hickey et al. (14).

## RESULTS

It was previously shown that Q fever and its endotoxemia were associated with increased phosphorylation of ribosomal protein S6 and with increased hepatic protein synthe-

sis (14). The participation of PKs in this phenomenon is now more closely examined. N were injected with 0.15 M NaCl, and L were injected with *C. burnetii* LPS. The initial average temperature ( $\pm$  standard deviation) of N injected with 0.15 M NaCl was  $38.5 \pm 0.1^\circ\text{C}$  ( $n = 5$ ); that of animals injected with LPS (L) was  $38.3 \pm 0.3^\circ\text{C}$  ( $n = 6$ ). There was no temperature change after 12 h for N, but there was an elevation to  $39.2 \pm 0.2^\circ\text{C}$  for L.

Identical postribosomal supernatant fractions prepared from N and L liver homogenates were reacted with intact N liver ribosomes. The reaction products were separated by gel electrophoresis with 10  $\mu$ g of reacted ribosomes per lane. After autoradiography, the S6 bands were excised and counted. The N samples had  $3,420 \pm 190$  cpm ( $n = 4$ ) while the radioactivity of the L bands was 70% greater ( $5,845 \pm 265$  cpm [ $n = 4$ ]), with greater kinase activity in the preparation from LPS-treated animals.

Further purifications (see Materials and Methods) of the N and L kinase again were performed in parallel. In all steps, the L-derived preparations maintained 40 to 50% greater activity than the controls (Fig. 1, Table 1).

**Substrate reactivities.** To compare this kinase with other classes of PKs, the preparations were reacted with protein substrates characteristic of particular kinase classes (7). Little kinase activity against dephosphorylated casein or  $\alpha$ S<sub>1</sub>-casein was observed, which distinguished the S6 kinase from the casein kinases. The preparations also had little activity against phosvitin, another acidic protein. Conversely, the N and L S6 kinases were each active against basic ribosomal proteins extracted with acetic acid from ribosomes. The enzyme preparations were active against mixed histones and H1, although not as much as against ribosomal proteins and H2B (Table 2); H2A and H3 were not significantly phosphorylated.

**Kinase agonists and antagonists.** Because major criteria for classifying PKs include dependency on or inhibition by certain effectors, the S6 kinase preparations were reacted with 80S ribosomes in the presence of known agonists or inhibitors of PK activity (Fig. 2). The N and L S6PKs were

TABLE 1. Partial purification<sup>a</sup> of PK from control and LPS-treated guinea pigs

Animal group and fraction	mg of protein/ml	Sp act <sup>b</sup>	Purification	% Increase (L/N)
<b>N</b>				
PRS <sup>c</sup>	0.7	431		
20–45% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.8	983	2.28	
DEAE peak II	0.32	3,478	7.98	
Glycerol gradient	0.03	55,044	127.7	
<b>L</b>				
PRS	0.7	613		42
20–45% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.8	1,425	2.32	45
DEAE peak II	0.32	5,543	9.04	59
Glycerol gradient	0.03	77,512	126.4	41

<sup>a</sup> All purification steps and assays were performed simultaneously. Assay procedures and reagents are described in the text. Reaction time per assay was 60 min.

<sup>b</sup> Picomoles of <sup>32</sup>P incorporated per milligram of protein.

<sup>c</sup> PRS, postribosomal supernatant.

not affected by 2 μM cAMP or by an excess of cAMP-dependent PK. The enzyme did not require Ca<sup>2+</sup> with phospholipid for activity and was not inhibited by sphingosine, which distinguished from the PK C group. Furthermore, mild treatment of the kinase with trypsin (see Materials and Methods) rapidly inactivated both the L and the less active N kinase preparations to the same low level, indicating that the enzyme is not a protease-activated kinase (24).

The enzyme was sensitive to 300 μg of heparin per ml and to 10 mM *N*-ethylmaleimide. The addition of Mg<sup>2+</sup> was necessary to maintain high activity, with an optimum concentration at 20 mM Mg<sup>2+</sup> (data not shown). Mn<sup>2+</sup> and Ca<sup>2+</sup> were unable to substitute for Mg<sup>2+</sup> and were inhibitory at relatively lower concentrations.

Another property of the enzyme is the poor ability of GTP to substitute for ATP as the phosphoryl donor in the kinase reactions. Unlabeled GTP was used as a competitor with 100 μM [ $\gamma$ -<sup>32</sup>P]ATP for phosphorylation of 80S ribosomes. Even at twice the concentration of ATP, GTP inhibited incorporation of labeled <sup>32</sup>P by only 30%. The apparent *K<sub>m</sub>* for ATP for both the N and L kinase preparations was 30 μM (Fig. 3). It should be noted that the apparent *V<sub>max</sub>* for the L preparation was nearly twice that of the N (Fig. 3), thereby

TABLE 2. Substrate reactivities of PK from control and LPS-treated guinea pigs

Substrate <sup>a</sup>	Sp act <sup>b</sup> in:		% Increase (L/N)
	N	L	
80S ribosomes	34.62	57.62	66
Ribosomal protein	37.01	63.58	72
Mixed histones	20.06	33.00	65
Histone H1	24.61	38.82	58
Histone H2A	10.48	13.63	30
Histone H2B	40.38	67.72	68
Histone H3	7.12	8.77	23
Dephosphorylated casein	5.54	5.29	-1
αS <sub>1</sub> -casein	3.08	3.27	1
Phosvitin	5.47	5.19	-1

<sup>a</sup> Final concentrations; mixed histones, phosvitin, dephosphorylated casein, and αS<sub>1</sub>-casein, 0.4 mg/ml; H1, H2A, H2B, and H3, 0.1 mg/ml; 80S ribosomes, 1 mg/ml. All systems used N and L preparations purified in glycerol gradients and were performed in parallel. Reaction time was 30 min. Assay conditions are described in the text.

<sup>b</sup> Nanomoles of <sup>32</sup>P incorporated per milligram of enzyme protein.

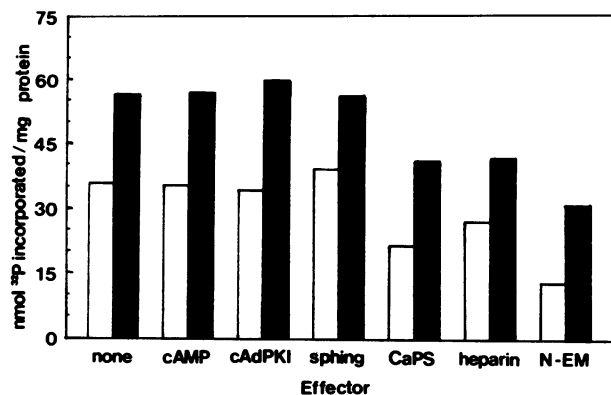


FIG. 2. Kinase reactivity with 80S ribosomes as affected by compounds used to classify PKs. Control and experimental enzymes had similar responses. Kinase activity was inhibited by Ca<sup>2+</sup> with phosphatidyl serine (CaPS), heparin, and *N*-ethylmaleimide (N-EM). Neither control nor experimental enzymes were affected by cAMP, cAMP-dependent PK (cAPKI), or sphingosine (sphing). Experimental conditions are described in Materials and Methods.

demonstrating a true stimulation of kinase activity during endotoxiosis.

**80S ribosome phosphorylation.** Specificity for proteins of intact ribosomes recognized by the PK was determined by reacting the enzyme with intact 80S ribosomes (see Materials and Methods). Basic ribosomal proteins were prepared from the reaction mixtures, separated by 2D electrophoresis, and autoradiographed (Fig. 4). Only two of the extracted proteins, L4 and S6, were phosphorylated to a significant extent; S6 was more highly phosphorylated. By allowing reactions to continue for various incubation times, it was possible to observe the appearance of sequentially phosphorylated derivatives of S6. In a 3-h reaction period, much of the <sup>32</sup>P label in the monophosphorylated derivative (Fig. 4A) appeared sequentially in the di- and triphosphorylated derivatives (Fig. 4B and C). This pattern of phosphorylation is similar to that previously seen when S6 had been labeled *in vivo* in response to Q fever and its endotoxiosis (Fig. 4D) (14). These results implicate the presently described PK in

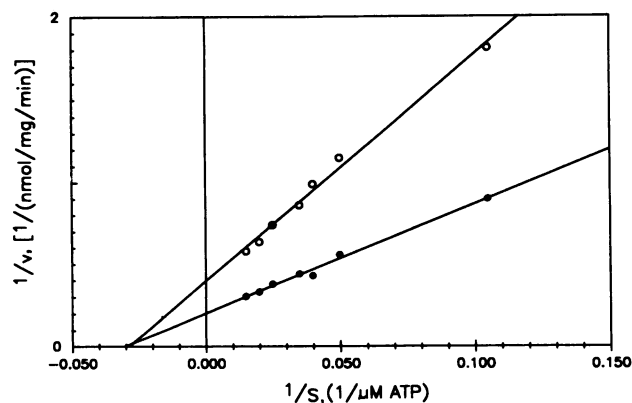


FIG. 3. Determination of apparent *K<sub>m</sub>* for ATP by Lineweaver-Burk analysis. The apparent *K<sub>m</sub>* for N and L preparations was 30 μM. In parallel analyses, the apparent *V<sub>max</sub>* for L (●) was almost twice that of N (○). Reaction mixtures (see Materials and Methods) were incubated for 15 min. Reaction velocity (*v*) is nanomoles of <sup>32</sup>P incorporated into 80S ribosomes per milligram of protein per minute. The ATP concentration (*S*) is micromolar.

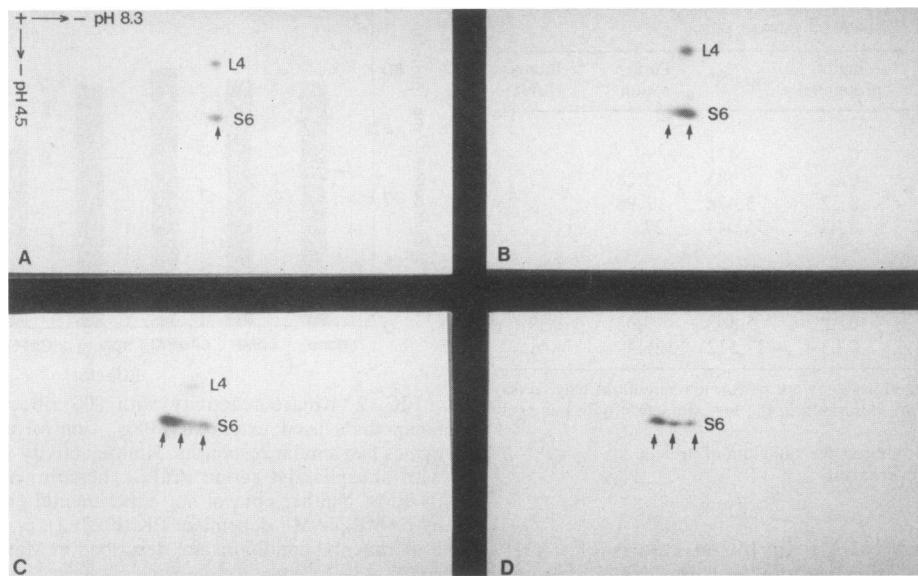


FIG. 4. (A, B, and C) Autoradiographs of ribosomal proteins separated by 2D gel electrophoresis after incubation of N ribosomes with S6PK for 60 min (A), 120 min (B), or 180 min (C). At 180 min, much of the  $^{32}\text{P}$  was present in the S6 species which contains at least three phosphoryl groups. This is comparable to the pattern previously found in vivo (D) (14).

the multiple phosphorylation of S6 in vivo (14) in response to infection and endotoxigenesis.

### DISCUSSION

The pathobiochemistry of Q fever and of the endotoxigenesis caused by the LPS of the rickettsial agent have common features of interest. Prominent among these are stimulated synthesis of protein and RNA (1, 26, 30). Enhanced RNA polymerase activities (22) and stimulated phosphorylation of nonhistone chromatin protein (9) are coincident, as are enhanced hepatic protein synthesis in vivo and phosphorylation of ribosomal protein S6 (14). Abundant evidence from other systems demonstrates the major roles of phosphorylations and dephosphorylations as regulatory mediators in the pathobiochemistry of disease (10). This paper reports an endotoxigenesis-stimulated PK which phosphorylates guinea pig ribosomal protein S6, indicating activation of phosphorylation systems during rickettsial infection. This kinase (S6PK) was partially purified, and its stimulated activity was retained through the purification steps. Insulin stimulates PK prepared and partially purified from 3T3-L1 cells by retaining stimulated activity through purification (6). Employing the criteria of Edelman et al. which characterize the classes of PKs (7), the S6PK presently described does not belong to the cAMP-dependent PKs. GTP fails to significantly replace ATP as a phosphoryl donor. The S6PK activity is independent of  $\text{Ca}^{2+}$  with phosphatidyl serine and thus does not belong to the PK C and calmodulin-dependent PK group. The S6PK is inactive against acidic casein and phosphotriester substrates and is thus eliminated from the casein I and II PK families. Unlike the Traugh protease-activated S6 kinase, the present S6PK is neither activated nor stimulated by the Traugh trypsin treatment (24). The S6PK phosphorylates at least three sites on S6. This activity is directly comparable to that reported previously of in vivo phosphorylation (14) and fits with the characteristics of the seryl-threonyl family of PKs (7). The S6PK has an apparent  $M_r$  of 55,000 by gel electrophoresis and silver staining (Fig. 5) and 68,000 by gel

filtration (Fig. 1C), and it resembles the S6PK of Tabarini et al. (27) and Erikson and Maller (8). The apparent  $K_m$  for ATP is  $30\ \mu\text{M}$  for the N and L S6PKs, which is comparable to  $K_m$  values reported for other PKs (8).

A model which relates phosphorylation events to key pathobiochemical events elicited by Q fever or by the LPS of its agent is proposed. It has been known for some time that protein and RNA synthesis in the liver are increased during infection or endotoxigenesis (9, 22, 26, 30). We have also observed increased plasma protein species of 14, 55, and 78 kilodaltons during endotoxigenesis and Q fever (M. Ershadi et al., unpublished data; W. D. Picking et al., *Fed. Proc.* **46**: 2084, 1987). We suggest that synthesis of these acute-phase proteins accounts for at least some of the observed protein increases. We further propose that stimulated transcription (22, 30) is coordinated with phosphorylation of nonhistone chromatin protein (9, 22). It is pertinent to note that stimulated RNA polymerase activities of rat hepatoma RNA polymerases I and II have been attributed to polymerase

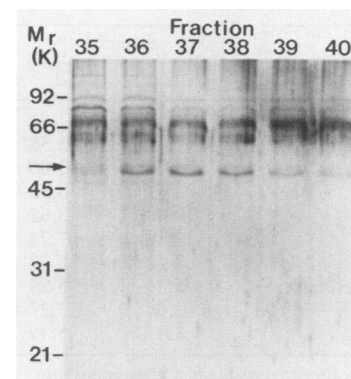


FIG. 5. Silver stain of S6PK preparations separated by gel filtration (Fig. 1C) and electrophoresed. The active S6PKs in fractions 36 through 38 have apparent  $M_r$ s of 68,000 by gel filtration and 55,000 (arrow) by gel electrophoresis.

phosphorylation (25). Increased ribosome numbers and mRNA species (26, 30) are products of stimulated transcription. Ribosomal protein S6 is phosphorylated with concomitantly increased protein synthesis (14; see also above). We propose that changes in phosphorylation of S6 during Q fever suggest activation of regulatory mechanisms during infection. These mechanisms involve protein phosphorylation-dephosphorylation reactions and serve to regulate a number of systems such as RNA synthesis, protein synthesis, and carbohydrate metabolism.

The relationship of S6 phosphorylation and protein synthesis remains uncertain (32). S6 phosphorylation by cAMP-dependent PK had no positive effect on ribosome-mRNA binding or translation in vitro (4, 21), and dephosphorylation of S6 in heat-shocked HeLa cells failed to alter translational activity of ribosomes in cell-free translation systems (28). In mouse myeloma cells, the kinetics of stimulated S6 phosphorylation during shifts of tonicity are different from changes in protein synthesis and suggest no obligatory relation between these two events (15).

On the other hand, S6 phosphorylation by protease-activated kinase II was directly correlated with increased translation when globin mRNA was used in in vitro systems (4, 21), and dephosphorylation of S6 in in vitro translation diminished (14). There is evidence supporting the concept that phosphorylated rat liver 40S subunits modify binding to mRNA (11, 29). Nevertheless, while many reports supporting a direct role of S6 phosphorylation in protein synthesis have appeared, the mechanism of such involvement has not been unequivocally established.

In any case, phosphorylation of S6 by protein kinases from diverse sources is stimulated by mitogens (8), insulin (13), protease activation (17), growth factors (23), and serum (2). The major feature of these systems is that measurement of this phosphorylation provides a significant clue for determining some of the regulatory mechanisms responsible for modified host metabolism. Likewise, stimulated S6 phosphorylation during endotoxiosis is an indicator of modulated cellular activity in response to trauma.

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