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Induction of hepatic synthesis of serum amyloid A protein and actin

(mRNA/recombinant DNA/acute-phase serum proteins)

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ABSTRACT **Major changes in the mRNA population of murine liver occur after administration of bacterial lipopolysaccharide, an agent that causes increases in the concentrations of acute-phase serum proteins. The mRNA for one of these, serum amyloid A, is increased at least 500-fold compared to the normal level. It becomes one of the most abundant hepatic mRNAs, and serum amyloid A synthesis comprises about 2.5% of total hepatic protein synthesis in the acute-phase response. Its synthesis is tissue-specific in that amyloid A mRNA was not detected in the kidney, an important site of amyloid fibril accumulation. The protein synthesized in largest amount by acute-phase liver tissue in culture is cytoplasmic actin. Its relative rate of synthesis is increased about 5-fold compared to the normal tissue; that of serum albumin is decreased to about one-third of its normal rate. The concentration of mRNA for serum albumin is decreased by a similar amount. Starting with induced liver RNA, we have constructed a recombinant plasmid containing most of the DNA sequence encoding the serum amyloid A polypeptide.**

In a number of mammalian species the concentrations of several proteins in the serum are increased as much as 1000-fold by infection, inflammation, or malignant neoplasia (1, 2). One of these acute-phase proteins is C-reactive protein, which is believed to play a role in defense against infection. It can fix complement in the presence of phospholipids or bacterial cell wall constituents, and it can cause opsonization for phagocytosis of various bacterial species (3). Another acute-phase protein is serum amyloid A (SAA). It is a possible precursor to the major protein component [amyloid A (AA)] of the predominantly extracellular amyloid fibrils found in the most frequent form of amyloidosis, reactive systemic amyloidosis. This condition is a serious complication of diseases involving chronic inflammation, one of which is rheumatoid arthritis (4). The individual polypeptide which is homologous to amyloid A protein is called SAAL (it is obtained by denaturation of SAA). The SAAL concentration may reach 1 mg/ml in serum by 18 hr after administration of bacterial lipopolysaccharide (LPS) or casein (1, 5, 6). Other abundant acute-phase serum proteins include the protease inhibitor α_2-acute-phase glycoprotein and the α_1-major acute-phase protein of the rat.

An attractive hypothesis to explain the rapid increase of SAAL concentration after LPS administration is induction of *de novo* biosynthesis by hepatocytes, followed shortly by release into the serum, as seen with C-reactive protein (2). ["Induction" is used to refer to specific stimulation of the relative rate of synthesis of a particular protein without implying an increase in synthesis of its mRNA (7)]. SAAL biosynthesis may provide a useful model for investigation of the control of gene activity in animal cells because its concentration in serum increases about 3000-fold (1).

However, conflicting evidence has been reported on the origin of SAAL. On the one hand, cultured hepatocytes produce material that binds to antibody against AA (8). Antigen has been detected in hepatocytes from animals treated with LPS but not in spleen cells or in normal liver cells (9). Furthermore, the acute-phase SAAL increase is prevented by inhibitors of RNA and protein biosynthesis (10).

On the other hand, it has been suggested that SAAL is not synthesized *de novo* in the acute-phase response but is released from a preexisting normal constituent of fibroblasts, kidney, aorta, leukocytes, etc. (11, 12). Determination of the tissue distribution and amount of SAAL mRNA during the acute-phase response should resolve some of the issues regarding amyloid protein production.

MATERIALS AND METHODS

Male Swiss mice were used (30–50 g). LPS (100 μg per animal) was dissolved in 0.15 M NaCl/4% mouse plasma and administered intraperitoneally. Rabbit antiserum to murine amyloid A protein was kindly provided by J. D. Sipe (1, 8, 10). Lipoproteins were removed (13) and IgG was purified (14). Purified human platelet actin was generously given by I. M. Herman and T. D. Pollard (15). Avian myeloblastosis virus reverse transcriptase was obtained from J. Beard (Division of Cancer Cause and Prevention, National Cancer Institute). Urea was ultrapure (Becton Dickinson).

Murine livers were frozen in liquid N_2. RNA was extracted and purified as described, except that guanidinium SCN stock solution was used throughout, in place of guanidine·HCl. This improved RNA recovery after LPS administration to 10 mg of RNA per g of liver, which is virtually all of the RNA present (16). RNA was then dissolved in 1% *N*-lauroylsarcosine/20 mM EDTA, pH 8, extracted with 2 vol of phenol/$CHCl_3$, 1:1 (vol/vol), ether extracted, ethanol precipitated, and stored at −20°C. Kidney RNA was purified by CsCl ultracentrifugation (16). Translation mixtures (25 μl) contained 2 μg of added RNA and 12 μCi of [^{35}S]methionine (1100 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) (17). For CNBr cleavage, 15 ^{3}H-labeled amino acids averaging about 25 Ci/mmol (Amersham, TRK.440) were used at 25 μCi total per 25 μl incubation mixture. Nonspecific precipitation with nonimmune IgG was omitted; 140 μg of IgG was incubated for 14 hr at 24°C with 9 μl of *in vitro* translation mixture (18), mixed for 3 hr with protein A-Sepharose (Pharmacia) in 1 M urea/0.1% Triton X-100/0.15 M NaCl/0.1 mM dithiothreitol/10 mM sodium phosphate, pH 7.2, and then

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Abbreviations: AA, amyloid protein A from deposits in tissues; SAA, native serum protein antigenically related to AA; SAAL, polypeptide antigenically related to AA, obtained by denaturation of SAA; LPS, lipopolysaccharide of Gram-negative bacteria (endotoxin); kDal, kdaltons.

washed three times with this solution. Cleavage by CNBr (40 mg/ml) was done in 70% formic acid at 26°C for 3 hr. Na$DodSO_4$ gel electrophoresis used acrylamide/*N,N'*-methylenebisacrylamide at 80:1 (19). Two-dimensional protein electrophoresis used pH 5–7 Ampholine (20). RNA was electrophoresed in 0.8% agarose gels containing formaldehyde, transferred to nitrocellulose, and hybridized with [^{32}P]DNA (21). Rat albumin plasmid pAlb576, kindly provided by J. M. Taylor, is similar to pAlbI (22) but has a larger rat DNA insert (2000 base pairs). Recombinant derivatives of pBR322 were obtained in *Escherichia coli* K-12 strain HB101; P1 containment was used.

Slices 0.5 mm thick and totaling 3 cm^2 were prepared from two fresh livers with a Stadie–Riggs microtome (A. H. Thomas), incubated for 60 min at 37°C in 1 ml of Eagle's minimal essential medium containing 150 μCi of [^{35}S]methionine (1100 Ci/mmol), frozen, thawed, and homogenized (7), sonicated twice for 30 sec at 0°C, and used for Na$DodSO_4$/polyacrylamide gel electrophoresis.

RESULTS

Synthesis of Apparent Precursor to SAAL in Wheat Germ-Derived Translation System. Translation of RNA extracted from murine liver 16 hr after LPS administration produced greater quantities of several polypeptides than did translation of normal liver RNA (Fig. 1). A 14-kilodalton (kDal) polypeptide was prominent among these. Other induced polypeptides were regularly observed at 24, 41, 55, and 56 kDal.

The 14-kDal polypeptide immunoprecipitated with IgG from rabbits immunized against murine amyloid A protein (Fig. 2),

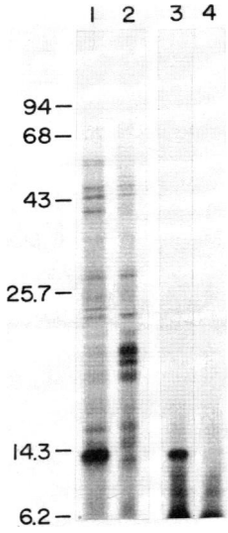

FIG. 1. *In vitro* translation of murine liver RNA extracted after LPS administration. The autoradiogram shows ^{35}S-labeled protein products electrophoresed in a 17.5% polyacrylamide gel (2 mm thick; run 15 hr at 14 mA). Sizes in kDal are shown. Lanes: 1, proteins synthesized with 16 hr post-LPS liver RNA as template; 2, proteins from normal liver RNA template; 3, products of 24 hr post-LPS RNA hybridized to recombinant plasmid pRS48 and eluted from it; 4, products of the same RNA adsorbed to a filter to which no DNA was applied and then eluted from it. The products of an incubation with no added RNA were similar to those shown in lane 4. The prominence of the 14 kDal polypeptide after *in vitro* translation (lane 1) does not imply that its mRNA is the most abundant after LPS administration. The wheat germ-derived translation system finishes small polypeptides more efficiently than large ones because it produces many incomplete peptides of the latter (18). A reticulocyte-derived system was not used because the large amount of endogenous globin comigrates with the 14 kDal polypeptide.

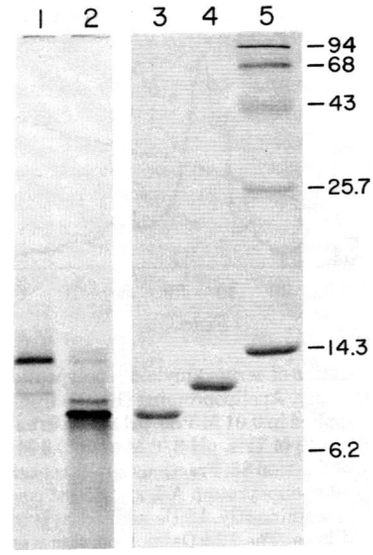

FIG. 2. Electrophoresis of purified SAAL, immunoprecipitated pre-SAAL, and CNBr cleavage products from them. Lanes: 4, SAAL purified by DEAE-cellulose chromatography (30 μg); 3, same after incubation with CNBr (55 μg); 1, anti-AA immunoprecipitate from *in vitro* translation of 16-hr post-LPS liver RNA (6000 cpm of ^{3}H-labeled protein); 2, as 1 but after incubation with CNBr (11,000 cpm of ^{3}H-labeled protein); 5, standards. Lanes 3–5 show proteins stained with Coomassie brilliant blue G-250; lanes 1 and 2 are a fluorogram of the same gel (25% polyacrylamide; 1 mm thick; electrophoresed 13 hr at 12 mA). Incomplete CNBr cleavage is the cause of some or all of the minor radioactive bands larger than 9.4 kDal in lane 2.

as SAAL does (1, 4–6). Nonimmune rabbit IgG bound only 3% as much 14-kDal polypeptide as anti-AA did. The immunoprecipitation was efficient in that the amount of anti-AA used for Fig. 2 specifically precipitated most of the 14-kDal protein from solution. This IgG preparation precipitated no visible band of radioactive protein when an identical experiment was done with normal murine liver RNA. The 14-kDal protein's mRNA was at least 500-fold more abundant in liver after LPS administration than in normal liver, as indicated by densitometry of the fluorograms (19).

SAAL is secreted into the serum *in vivo*. When a secreted polypeptide is synthesized *in vitro* without microsomes, a larger precursor is usually produced, with additional amino acid residues at its NH_2 terminus (a signal peptide). We tested this possible relationship of SAAL to the 14-kDal polypeptide.

Only one polypeptide was observed when the purified SAAL (Fig. 3) was subjected to Na$DodSO_4$/polyacrylamide gel electrophoresis (Fig. 2). Its apparent size (12 kDal) and composition were as expected (24). SAAL contains two methionine residues, at positions 16 and 23 of the sequence (5, 6, 24). Consequently, CNBr cleavage should yield a major COOH-terminal peptide of about 9.4 kDal. This was observed (Fig. 2). The other two peptides are too small (1.5 and 0.8 kDal) to be readily visualized by Na$DodSO_4$/polyacrylamide gel electrophoresis.

If the 14-kDal immunoprecipitable protein made *in vitro* were similar to SAAL except for additional residues at its NH_2-terminus, it should yield the same COOH-terminal peptide upon CNBr cleavage. This was found (Fig. 2); the major radioactive peptide comigrated with the COOH-terminal peptide of SAAL. We conclude that the 14-kDal protein is similar to SAAL but has additional residues at its NH_2-terminus, based on immunoprecipitation and CNBr cleavage results. SAAL was abundant after 1 hr of radioactive labeling of post-LPS liver tissue (see below), but the 14-kDal protein has been found only after translation *in vitro*. These results are expected for a secreted

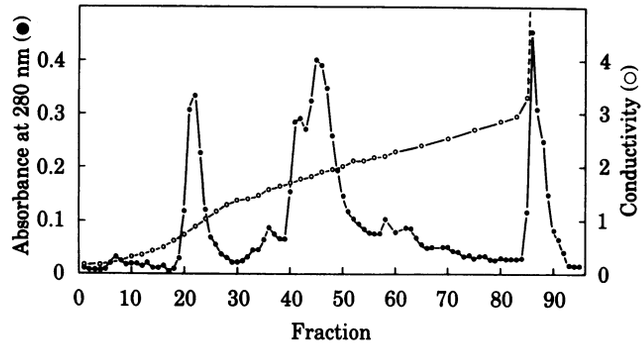

FIG. 3. Purification of serum amyloid A polypeptide by DEAE-cellulose chromatography. Apolipoproteins (13, 23) from 6.3 ml of plasma (15 mg) were applied in 0.01 M Tris, pH 8/6 M urea and eluted with a gradient of 0.01–0.10 M Tris, pH 8/6 M urea; 0.5 M Tris, pH 8/6 M urea was applied at fraction 83. Fractions 20–24 contained SAAL (Fig. 2), 41–50 contained apolipoprotein A-I, and 85–88 contained polypeptides <12 kDal, predominantly. Little additional protein eluted with 0.1 M NaOH/6 M urea. The 12-kDal polypeptide was not seen when apolipoproteins of normal plasma were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis.

protein from which a signal peptide is cleaved during its synthesis *in vivo*.

The concentration of SAAL in murine serum peaks 16–24 hr after administration of 50 μg of LPS intraperitoneally; it then declines quickly to about 10% of the peak concentration by 48 hr (1). SAAL exits rapidly from the liver after its synthesis (8, 9). These facts indicate that the SAAL mRNA concentration must also peak at about 16 hr after administration of LPS, unless SAAL synthesis is subject to control at the level of translation. To test this prediction, we extracted hepatic RNA at various times after administration of LPS and incubated it in the wheat germ-derived translation system. The synthesis of 14-kDal polypeptide was much greater upon translation of RNA extracted 9–24 hr after administration of LPS compared to the translation of RNA extracted from normal liver or from liver 69 hr after LPS (Table 1). The results indicate a broad peak in SAAL mRNA concentration from 9 to 24 hr after administration of LPS, suggesting that translation-level control does not play a major role in SAAL synthesis.

Table 1. mRNA for 14-kDal polypeptide as a function of time after LPS administration

Time after LPS, hr	Induced 14-kDal polypeptide, %	Relative amount of SAAL mRNA
No LPS	0	<0.01
2.7	2.6	0.16
9	13	0.82
16	12	0.74
24	16	1.00
43	3.9	0.25
69	0.4	0.03

RNA was extracted from livers of two mice at the times indicated after administration of 50 μg of LPS. After *in vitro* translation and $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1), fluorograms were obtained that were within the linear range of exposure as a function of radioactivity (19). The amount of SAAL mRNA in normal liver was 1/500th or less of the amount 24 hr after LPS (see text). The center column shows the amount of ^{35}S-labeled proteins between 13.5 and 14.5 kDal as a percentage of total ^{35}S-labeled proteins; 7.0% (the amount found with no LPS) has been subtracted from each value. No significant change in total mRNA template activity has been found after LPS administration.

Induced Proteins Comprise a Large Portion of Hepatic Protein Synthesis After LPS Administration. Incubated liver slices have been shown to give valid estimates of *in vivo* protein synthesis rates (7, 26). They synthesized a larger amount of 12-kDal protein after LPS administration (Fig. 4*A*). Immunoprecipitation showed that most of the 12-kDal protein made after administration of LPS was SAAL (Fig. 4*B*); it comprised 2.5% of the radioactive protein (19). Little or no SAAL was made by normal liver (Fig. 4*D*).

The hepatic protein made in largest amount after administration of LPS has an apparent molecular weight of 41,000 (Fig. 4). This protein comigrated with the major cytoplasmic actin of human platelets on $NaDodSO_4$/polyacrylamide gel electrophoresis in 10% and 20% gels. It comprised about 10% of the ^{35}S-labeled protein synthesized from 22 to 23 hr after administration of LPS (Figs. 4 and 5). Hepatocytes contain actin (20). The ^{35}S-labeled proteins synthesized in culture were further analyzed by two-dimensional electrophoresis. The protein made in largest amount after administration of LPS comigrated precisely, in both dimensions, with nonradioactive actin purified from platelets (Fig. 5*B*). Actin represented about 2% of the radioactive protein made by normal liver tissue (Fig. 5*A*).

Several other proteins whose relative rates of synthesis are increased by LPS are evident, at about 55, 66, and >68 kDal (Figs. 4 and 5). Polypeptides whose relative synthesis rates are increased severalfold or more appear to comprise >30% of hepatic protein synthesis at 22–23 hr after administration of LPS. The synthesis rates of some polypeptides must be correspondingly diminished after LPS administration. Serum albumin is among these. It forms the most intensely exposed spot at about

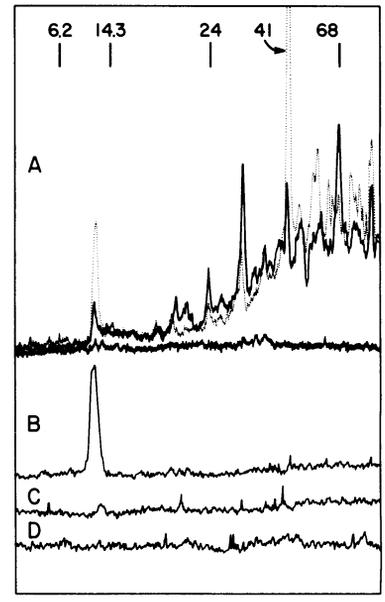

FIG. 4. Immunoprecipitation and electrophoresis of radioactive proteins synthesized by liver tissue after LPS administration. The figures show densitometer tracings of fluorograms of ^{35}S-labeled proteins. (*A*) Proteins synthesized during a 1 hr incubation by normal liver tissue (solid line) or liver tissue 22 hr after administration of LPS (dotted line). The unexposed film baseline density is also shown (solid). (*B* and *D*) Protein immunoprecipitated by anti-AA, from homogenate of tissue after LPS administration (*B*) or of normal tissue (*D*). (*C*) Protein precipitated by normal rabbit IgG from homogenate of tissue after LPS administration. The 20% acrylamide gel (1 mm thick) was electrophoresed for 9 hr at 2 W (19). The methionine content of SAAL, 1.8%, is identical to that of total hepatic protein (data not shown), and similar to that of many well-characterized proteins. The results were unaltered when the tissue was incubated *in vitro* for 1 hr prior to the 1-hr labeling period. Sizes are shown in kDal.

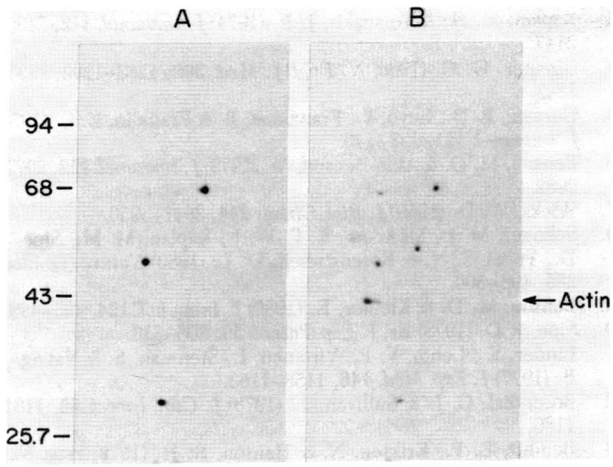

FIG. 5. Two-dimensional electrophoresis of ^{35}S-labeled proteins synthesized by liver tissue in culture for 1 hr. (*A*) Normal liver; (*B*) liver 22 hr after administration of LPS. Homogenates containing 45 μg of protein and 9300 (*A*) or 4200 (*B*) cpm of ^{35}S-labeled proteins were analyzed by isoelectric focusing followed by NaDodSO$_4$/polyacrylamide gel electrophoresis (20). The basic end was at the right side of each fluorogram. These are short exposures, for quantitation of the proteins synthesized in largest amounts. The radioactive protein in the culture medium (<10%) does not significantly alter the distribution shown here.

68 kDal in Fig. 5 (27). It comprised 7–11% of the ^{35}S-labeled protein made by normal murine liver in culture and about one-third as much after administration of LPS (Figs. 4 and 5) (22).

SAAL mRNA Increases in Amount After LPS Administration, and Albumin mRNA Decreases. We used recombinant plasmid DNAs to learn whether the changes in rates of synthesis of SAAL and albumin are attributable to changes in amounts of their mRNAs or to translational activities of those mRNAs. To isolate a plasmid containing SAAL encoding sequences, we purified polyadenylylated RNA from murine liver 24 hr after administration of LPS. Double-stranded DNA was prepared by use of reverse transcriptase and DNA polymerase I (28). Homopolymer dC tails were added to it, and it was cloned by using the *Pst* I site of pBR322 (29). The resulting recombinant plasmids (142 of them) were screened for efficiency of hybridization with reverse transcripts of normal liver RNA and of RNA from liver after administration of LPS (25). Nine plasmid DNAs that hybridized much more efficiently with the latter were denatured and bound to nitrocellulose. After annealing with RNA from liver after LPS adminstration, the hybridized RNA was eluted and translated in the wheat germ-derived system (30). Plasmid pRS48 yielded a 14-kDal polypeptide (Fig. 1) that immunoprecipitated specifically with anti-AA. Most of the other recombinant plasmids did not yield the 14-kDal polypeptide by this method. pRS48 contains a 480-base-pair segment bounded on each side by a *Pst* I site, in addition to its pBR322 DNA sequences. We conclude from these and similar results that pRS48 encodes SAAL.

Normal and LPS-treated murine liver RNAs were tested for their ability to hybridize with pRS48 DNA, by RNA filter transfer hybridization (21). After LPS administration, liver contained a RNA that hybridized specifically with pRS48; it was not detected in normal liver (Fig. 6). Because it coelectrophoresed with 9S rabbit globin RNA in this denaturing gel, its length is about 650 nucleotides, including poly(A). The results indicate that the amount of SAAL mRNA increases after LPS administration, not merely its activity in translation.

To learn whether the diminution of albumin synthesis is explained by a decrease in albumin mRNA concentration after

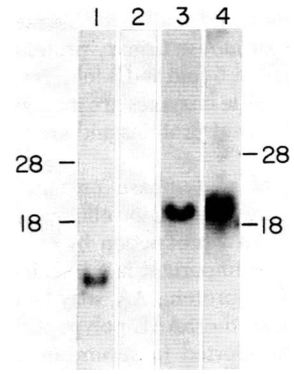

FIG. 6. Plasmid DNA hybridization with RNA transfer filters. Lanes 1 and 2, [^{32}P]DNA of SAAL-encoding plasmid pRS48. Lanes 3 and 4, [^{32}P]DNA of albumin plasmid pAlb576. Lanes: 1, total liver RNA (7 μg) from a mouse 24 hr after administration of LPS; 2, RNA (7 μg) from a normal mouse liver; 3, total liver RNA (20 μg) pooled from six mice 12 hr after administration of LPS; 4, RNA (19 μg) from six normal mice. These autoradiograms are representative of three experiments. RNA extracted 16 hr after administration of LPS gave a result similar to that of lane 3. Positions of 28S and 18S ribosomal RNAs are shown.

administration of LPS, we used ^{32}P-labeled pAlb576, a rat albumin plasmid DNA. One predominant band of RNA hybridizing with pAlb576 was found, with the expected mobility (Fig. 6) (22). One-third as much plasmid DNA hybridized to RNA from liver after LPS administration as to normal liver RNA. The amount hybridized was proportional to the amount of RNA applied. Normal liver and LPS-treated liver yield the same amount of total RNA. Consequently, the decrease in the amount of albumin mRNA after administration of LPS matches the diminution of the relative rate of albumin synthesis.

SAAL mRNA is Tissue-Specific. Because SAAL might be synthesized by many cell types, we examined the amount of SAAL mRNA in the kidney (amyloid fibrils are frequently found there). No SAAL mRNA was detected by anti-AA immunoprecipitation of translation products of kidney RNA. The RNA was extracted during the period of increasing SAAL concentration, at both 7 and 10 hr after administration of LPS (1, 10). Densitometry of the fluorograms indicated that the kidney has less than 1/50th of the liver's SAAL mRNA. Furthermore, no major change in the kidney mRNA population was observed after LPS administration.

DISCUSSION

The results described here conform to the rule that differential synthesis of eukaryotic proteins usually results from differences in mRNA concentrations (e.g., refs. 16 and 22). We have shown that increased SAAL synthesis after LPS administration reflects a very large increase in SAAL mRNA (at least 500-fold). Furthermore, the decrease to one-third in serum albumin's relative synthetic rate reflects the decrease, to one-third, of its mRNA. In contrast, heat shock protein synthesis in *Drosophila* is a counterexample to the rule: it involves a clear example of translational control (31). (The mRNAs for *Drosophila* proteins translated at 25°C remain at 36°C but are not translated at 36°C *in vivo*.) A major role for translational control in the acute-phase response is not indicated by our results.

The induction of acute-phase serum proteins and actin is among the largest and most rapid changes known in eukaryotic protein synthesis. We speculate that SAAL mRNA may increase more than 3000-fold, like the amount of SAAL polypeptide (1). Other inducible proteins can be made in large amounts by the liver. α_{2u}-Globulin comprises 1% of protein synthesized by

male rat liver; it is not found at all in female rats (32). The ranges of modulation of major mouse urinary proteins and metallothioneins are much smaller, 5-fold and 3-fold, respectively (33, 34). Also, a number of hepatic enzymes are increased by hormones, but they are not abundant proteins and are induced 10-fold or less (7).

The mechanism of acute-phase protein induction is not known in detail. However, a factor elicited from macrophages by LPS stimulates SAAL production by cultured hepatocytes (8). This factor may be important in SAAL induction *in vivo*.

The amyloid A fibril protein, AA, may be produced by proteolytic cleavage (4) of the SAAL polypeptide synthesized by hepatocytes and transported in serum amyloid A to sites of amyloid deposition such as the kidney (8, 9, 13). Our findings support this model. The amount of SAAL synthesized by the liver appears to be comparable to that found in blood plasma. Of course, other tissues may also synthesize SAAL. The recombinant plasmid described here should permit sensitive measurements of SAAL mRNA outside the liver.

Coelectrophoresis in two dimensions with human platelet actin is strong evidence that the protein synthesized in largest amount by cultured liver after administration of LPS is cytoplasmic actin. Coelectrophoresis is not unexpected because the amino acid sequence of cytoplasmic β-actin appears to be invariant among mammals (35). This is the main form of actin in rat hepatocytes and human platelets (20). The increase in actin synthesis may be related to changes in the liver's secretion activities. Cytochalasin D, a relatively specific inhibitor of actin microfilament function, interferes with lipoprotein secretion by cultured hepatocytes (36).

The magnitude of the induction of SAAL mRNA should facilitate investigation of the control of its synthesis. Also, the recombinant plasmid described may permit determination of the number of genes encoding SAAL. The nucleotide sequence indicates that the plasmid contains most of the residues encoding SAAL (unpublished data). The sequence of murine SAAL from inbred strains shows more than one amino acid residue at several positions, indicating that at least four genes encode similar polypeptides (5, 24). For comparison, the family of major urinary proteins of the mouse, also synthesized in the liver, is encoded by about 15 genes (33).

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