Synthesis and Regulation of Acute Phase Plasma Proteins in Primary Cultures of Mouse Hepatocytes

HEINZ BAUMANN, GERALD P. JAHREIS, and KATHERINE C. GAINES
Department of Cell and Tumor Biology, Roswell Park Memorial Institute, Buffalo, New York 14263

ABSTRACT Adult mouse hepatocytes respond in vivo to experimentally induced acute inflammation by an increased synthesis and secretion of α_1-acid glycoprotein, haptoglobin, hemopexin, and serum amyloid A. Concurrently, the production of albumin and apolipoprotein A-1 is reduced. To define possible mediators of this response and to study their action in tissue culture, we established primary cultures of hepatocytes. Various hormones and factors that have been proposed to regulate the hepatic acute phase reaction were tested for their ability to modulate the expression of plasma proteins in these cells. Acute phase plasma and conditioned medium from activated monocytes influenced the production of most acute phase plasma proteins, and the regulation appears to occur at the level of functional mRNA. Purified hormones produced a significant anabolic response in only a few cases: dexamethasone was found to be effective in maintaining differentiated expression of the cells; and glucagon produced a specific inhibition of haptoglobin synthesis. When cells were treated with a combination of conditioned monocyte medium and dexamethasone, secretion of proteins was markedly reduced. The carbohydrate moieties of all plasma glycoproteins were incompletely modified, apparently as a result of decreased intracellular transport of newly synthesized plasma proteins. Although primary hepatocytes were not phenotypically stable in tissue culture, the cells nevertheless retained a broad response spectrum to exogenous signals. We propose this as a useful system to study the production of plasma proteins and thereby pinpoint the nature and activity of effectors mediating the hepatic acute phase reaction.

Mammalian liver responds to various systemic injuries, such as acute inflammation, infection, or burns with profound changes in the activity of a number of genes (21). This reaction of the liver is expressed as the increased synthesis and secretion of several plasma proteins, the acute phase reactants (20). The quantitative and qualitative pattern of the hepatic acute phase response is to some degree species specific. For instance, the major acute phase reactant in humans and rabbits is C-reactive protein (1, 3), in humans and mice serum amyloid A (25), and in rats α_1-acid glycoprotein (AGP[1]) and α_2-macroglobulin (22, 27). Numerous attempts have been made to identify the biochemical nature and the mode of action of the effector(s) mediating the hepatic acute phase response (22). From measurements of the concentrations of circulating hormones during the course of acute inflammation, several potential effectors, such as glucocorticoids, glucagon, or growth hormone have been suggested (9, 12, 16, 17). Furthermore, the observation that mononuclear cells play an essential role in the initial stages of the acute phase (13, 34, 37) suggests that components released by these cells might also contribute to the response of the liver (20, 31, 35).

An unambiguous assessment of the modulating activity of the proposed factors has been limited in part by the absence of a tissue culture system permitting analysis of the entire spectrum of the hepatic response. Recent studies, however, suggest that the regulation of a few acute phase plasma proteins can be reproduced to some extent in tissue culture. For example, we have recently shown in primary rat hepatocytes that dexamethasone modulates the synthesis of some major acute phase proteins, including α_1-AGP and α_2-macroglobulin similar to their regulation in vivo during experimentally induced inflammation (6). Further analyses have indicated that corticosteroids are not the sole inducers of these proteins during the acute phase reaction. The use of leukocyte-derived factors on primary rat hepatocytes has revealed a stimulation of the production of fibrinogen, a less prominent acute phase protein (29). A similar study using primary cultures of mouse

[1] *Abbreviations used in this paper:* α_1-AGP, α_1-acid glycoprotein; SAA, serum amyloid A.

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hepatocytes by Selinger et al. (30) demonstrated that monokines stimulate the synthesis and accumulation of serum amyloid A (SAA).

The above studies have been limited to measurements of one or two of the acute phase plasma proteins. To learn more about experimental regulation of the entire spectrum of acute phase plasma proteins, we have analyzed the secretion products of primary mouse hepatocytes treated in tissue culture with several of the factors implicated in mediation of the hepatic acute phase reaction. We report here that primary cultures of mouse hepatocytes, although not in a stable, differentiated state, are still capable of responding to various treatments, such as monocyte factors and acute phase plasma by a coordinate change in production of acute phase plasma proteins. The observed anabolic response of hepatocytes to glucagon, acute phase serum, or macrophage factors combined with dexamethasone was unexpected, and may provide some insight into the intricate regulatory events operating in vivo.

MATERIALS AND METHODS

Animals: Hepatocytes and activated monocytes were obtained from 2–4-mo-old males of the C57Bl/6J mouse strain. Acute inflammation was evoked by two subcutaneous injections of turpentine (50 μl/25 g body weight) in the lumbar region of an animal. Acute phase blood was obtained by heart puncture 12 h after initiation of inflammation. Plasma was prepared from heparinized blood by centrifugation for 25 min at 2,000 *g*. Serum was collected from blood that was allowed to coagulate for 1 h at 25°C followed by 18 h at 4°C.

Cells and Tissue Culture Conditions: Mouse hepatocytes were prepared as described in detail elsewhere (K. C. Gaines and D. Doyle, manuscript in preparation). Briefly, the liver was perfused in situ with collagenase through the inferior vena cava. The total cells released were subjected to three rounds of centrifugation for 3 min at 50 *g* to enrich the parenchymal cell population to >95%. The viability of the cells was routinely >97%. The yield ranged from 7–20 × 10^6 cells per liver. The hepatocytes were suspended at a density of 5 × 10^5 cells/ml in Dulbecco's minimum essential medium containing 4.5 g/liter glucose, 10% heat-inactivated fetal calf serum, and 2 mM glutamine. 2 ml of cell suspension per 10 cm^2 culture area were placed in collagen-coated dishes and after 1 h at 37°C, the nonadherent cells were removed by replacing the medium. At this time (designated as 0 h of the experiment), hormones or factors were added to the culture at following concentrations: mouse or rat plasma or serum, 20%; dexamethasone, 0.01–1 μM; glucagon, 10 μM; insulin, 50 mU/ml; somatomedin C, 10 ng/ml; thyroxin, 10 μg/ml; bovine growth hormone, 10 μg/ml; epidermal growth factor, 1 μM; dihydrotestosterone, 10 μM; and 8-Br-cAMP, 0.1–1 mM. The cells were maintained at 37°C under a 5% CO_2/95% air atmosphere. The culture media were changed daily.

Inflammatory monocytes were induced by an intraperitoneal injection of 1 ml of 4% (wt/vol) thioglycolate. Three days later, the monocytes were collected by peritoneal lavage with an average yield of 2–3 × 10^7 cells/mouse. The cells were washed once and placed in monolayer culture (4 × 10^7 cells/25 cm^2). The adherent cells were cultured for 18 h in 3 ml/25 cm^2 Dulbecco's minimum essential medium containing 10% fetal calf serum and 10 μg/ml lipopolysaccharide (extracted from Escherichia coli by trichloroacetic acid, serotype 0127:B8, Sigma Chemical Co., St. Louis, MO). The conditioned monocyte medium was centrifuged for 10 min at 1,500 *g* and the supernatant fraction diluted with ⅓ vol of fresh culture medium. This preparation was used immediately for treatment of hepatocytes.

Radioactive Labeling and Cell-free Translation of RNA: The cells were washed three times with serum-free Dulbecco's minimum essential medium containing 10% of the usual concentration of methionine. For a 10 cm^2 cell monolayer, 1 ml of the same medium plus 50–150 μCi [^{35}S]methionine (1,000–1,200 Ci/mmol) was added and incubated for 6 h. The medium was then removed and cleared of any released cells by centrifugation for 5 min at 1,000 *g*. Cell monolayers were washed three times with phosphate buffered saline and used for measurements of cellular proteins (see Tables). Aliquots from the medium (25–50 μl containing 2–4 × 10^5 acid-insoluble cpm) were used without further treatment for polyacrylamide gel electrophoresis. For crossed immunoelectrophoretic analysis, 500 μl of labeled culture medium was dialyzed for 24 h against 50 mM NH_4HCO_3, freeze-dried, and redissolved in electrophoresis buffer (4). Total RNA was extracted from liver or tissue culture cells and translated in a cell-free system as described previously (6).

Analysis of Secreted Hepatocyte Proteins: The following plasma proteins were purified from labeled hepatocyte medium employing published procedures: haptoglobin (18), α_1-AGP (15, 38), and hemopexin (32). Two-dimensional electrophoresis was carried out as described by O'Farrell (28). Second dimensions consisted of uniform 11% polyacrylamide gels. The radioactive pattern was visualized by fluorography (10). The protein spots were identified by the following methods: albumin and transferrin by immunoprecipitation; major urinary protein, α_1-antitrypsin, α_1-antichymotrypsin, and apolipoprotein A-I by cell-free translation of mRNA selected by the corresponding cDNA probes; haptoglobin, hemopexin, α_1-AGP, α_1-antitrypsin, and α_1-antichymotrypsin by biochemical purification; and β-fibrinogen, antithrombin III, and SAA by comparison with published characterizations (2, 26). To determine the radioactivity present in the separated proteins, spots were cut from the dry gel according to the fluorographic image. Neighboring areas of the gel not containing detectable spots served as background. The gel pieces were rehydrated in 100 μl water, incubated for 18 h with 1 ml of NCS solubilizer (Amersham Corp., Arlington Heights, IL). After addition of 10 ml toluene-based, acidified scintillation fluid, radioactivity was determined by a liquid scintillation counter.

Crossed immunoelectrophoresis of the proteins secreted by hepatocytes was performed according to the procedure of Weeke (36). The second dimension gels contained 2% rabbit anti-mouse acute phase serum. The precipitin lines were visualized by autoradiography. The identity of the antigens were determined by separation of the radioactive materials recovered from individual precipitin lines on two-dimensional polyacrylamide gels (5).

RESULTS

Synthesis and Secretion of Acute Phase Plasma Proteins by Primary Hepatocytes

To ascertain which plasma proteins are affected in their synthesis and secretion by experimentally induced inflammation in vivo, we prepared hepatocytes from normal animals and animals that had undergone 24 h of inflammatory stress, immediately cultured the cells in medium containing [^{35}S]-methionine for 6 h, and collected the labeled secretory proteins. The two-dimensional gel analysis of the secretory proteins depicted in Fig. 1 reveals that four protein species, α_1-AGP, haptoglobin, hemopexin, and SAA exhibit a marked increase in relative synthetic rate as reflected by their radioactivities. The production of two other proteins, albumin and apolipoprotein A-I, is substantially reduced. To verify that these changes are a general phenomenon of the mouse liver, we analyzed five additional pairs of independent cell preparations. In all cases, the above noted plasma proteins were consistently altered as shown in Fig. 1 (for quantitation see below, Tables I–III). This defines a minimal set of major positive acute phase plasma proteins which can be recognized on the level of cellular synthesis and secretion, which is composed of α_1-AGP, haptoglobin, hemopexin, and SAA. Inspection of electrophoretic patterns in Fig. 1 reveals additional differences in production of other unidentified secretory proteins. However, these changes are either less pronounced or less reproducible in different cell preparations and are not further studied.

Responsiveness of Hepatocytes in Tissue Culture

To alter the secretion program of hepatocytes by addition of a restricted set of hormones or factors to a basic culture medium is a preferred approach to specifically define essential mediators of the hepatic acute phase reaction. Nevertheless, the use of such culture milieus, may be an inadequate representation of the in vivo environment. To establish the reaction potential of primary hepatocytes, it is necessary to demonstrate that in tissue culture, the cells, following treatment with various effectors found in an inflamed animal, maintain responses that are qualitatively and quantitatively comparable

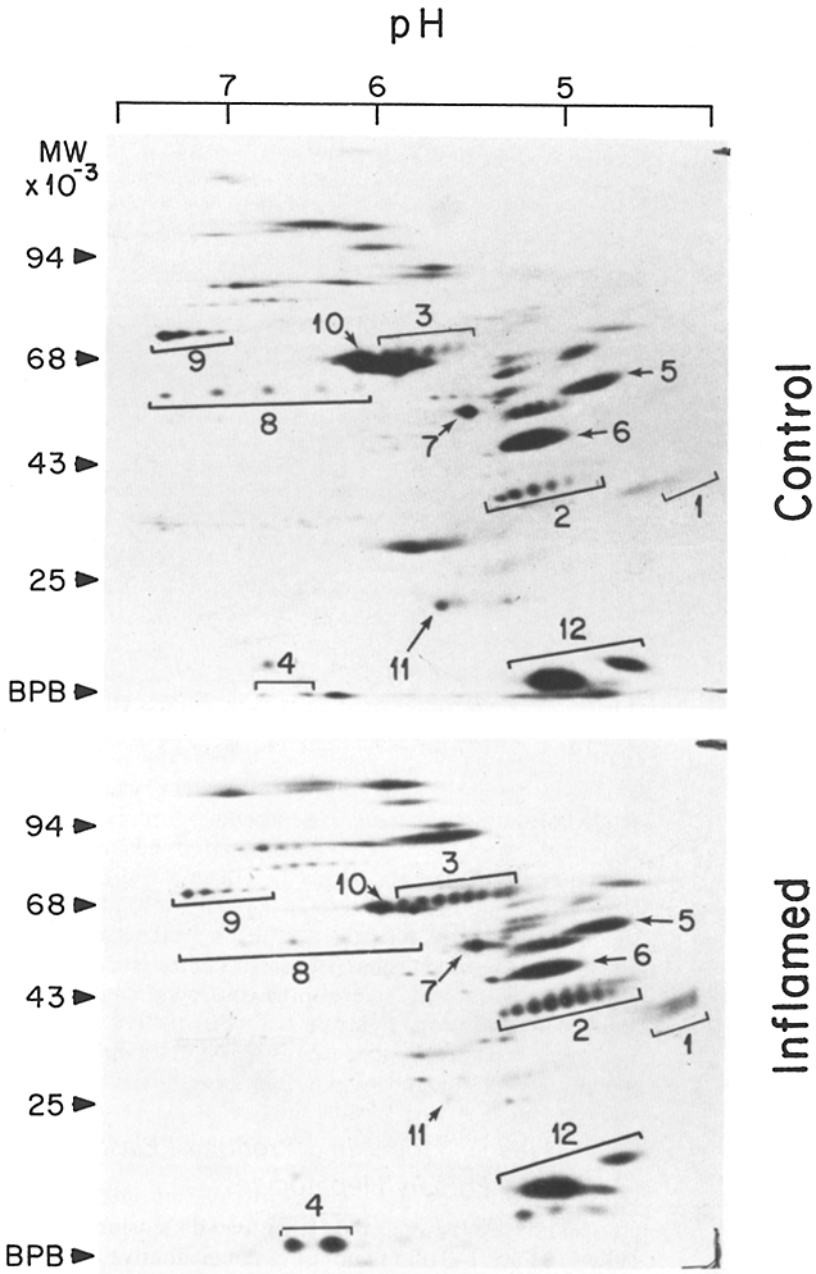

FIGURE 1 Effect of an acute inflammation on synthesis and secretion of plasma protein by hepatocytes. Hepatocytes were prepared from a normal male mouse (*control*) and from a mouse 24 h after subcutaneous injection of turpentine (*inflamed*). 1 h after placing in monolayer culture, the cells were labeled for 6 h with [^{35}S]methionine (150 μCi/ml). 50 μl of each culture medium was separated by two-dimensional polyacrylamide gel electrophoresis. The fluorograms were exposed for 18 h. Spots indicated with numbers represent the following proteins (same numbering system was used for all subsequent figures): *1*, α_1-acid glycoprotein; *2*, β-haptoglobin; *3*, hemopexin; *4*, serum amyloid A; *5*, α_1-antichymotrypsin; *6*, α_1-antitrypsin; *7*, antithrombin III; *8*, β-fibrinogen; *9*, transferrin; *10*, albumin; *11*, apolipoprotein A-1; *12*, major urinary proteins. *BPB*, bromphenol blue.

with those seen in vivo. Therefore, we used plasma or serum from animals 12 h after induction of the acute phase. These blood fractions should contain the humorally transmitted signals for the hepatic reaction. Hepatocytes cultured in the presence of acute phase plasma (Fig. 2) retain the overall pattern of secretion found in cells at 0 h (see Fig. 1), yet the hepatocytes have an enhanced synthesis of the major acute phase proteins: α_1-AGP, haptoglobin, hemopexin, and SAA, and a significant reduction in albumin. In parallel experiments, where hepatocytes have been maintained in medium containing normal mouse plasma (data not shown), we have observed preservation of the initial pattern of plasma protein production. Some proteins, such as α_1-antichymotrypsin and β-fibrinogen, were synthesized in relatively smaller amounts than by 0-h cultures (Fig. 1) or cells in acute phase plasma (Fig. 2), but in no case was there any significant increase in the synthesis of the four major acute phase proteins.

When serum from inflamed animals was used instead of plasma, the effects were similar though less pronounced. Serum from normal or inflamed mice or rats, however, produces a marked inhibition of haptoglobin synthesis. This effect was not observed when fetal calf serum (Fig. 3) or horse serum (data not shown) was used. In addition, two new proteins, *a* and *b* in Fig. 2, were seen in variable amounts as a function of time in culture and this irrespectively of the origin of plasma or serum used.

Having demonstrated that primary mouse hepatocytes are capable of modifying the production of the major acute phase plasma proteins in tissue culture as a consequence of the above treatments, we then probed the response of the cells to specific hormones or factors, all of which are proposed to be involved in regulation of the hepatic acute phase reaction. To do so, we examined the proteins synthesized and secreted following 48-h treatment with insulin, epidermal growth factor, somatomedin C, growth hormone, thyroxine, dihydrotestosterone, and combinations thereof. Repeated determinations showed no specific effects from these factors. The presence of lipopolysaccharides in the medium was also found

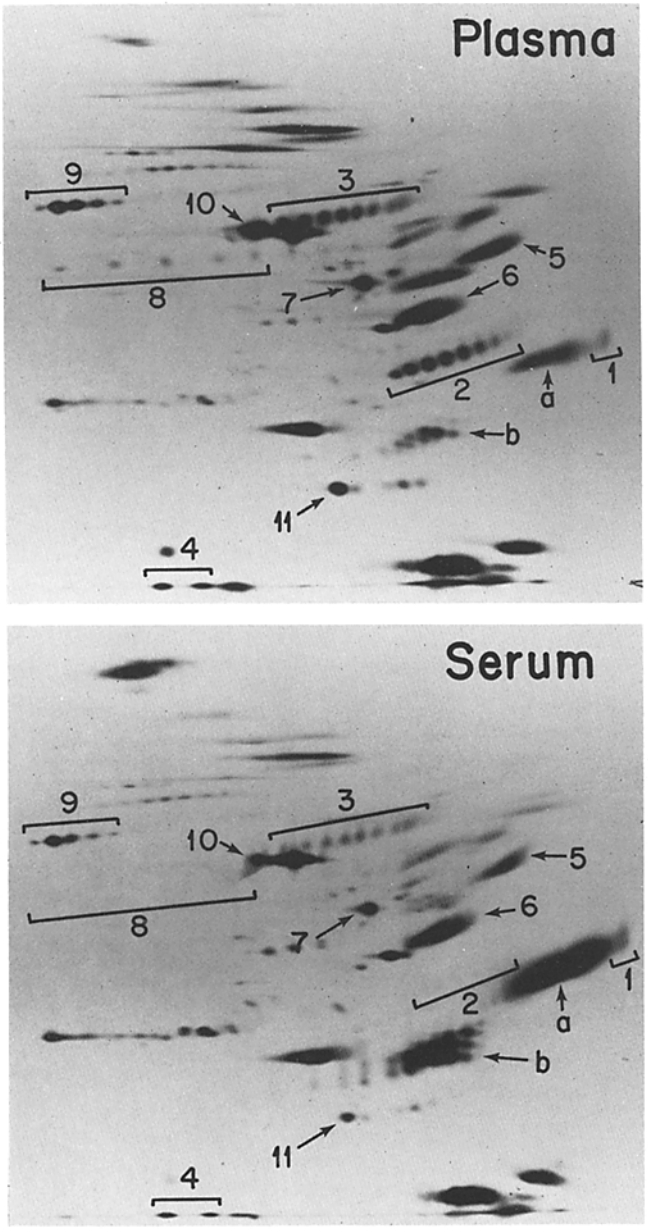

FIGURE 2 Influence of acute phase plasma and serum on hepatocytes. Plasma and serum were collected from a single mouse 12 h after injection of turpentine. Hepatocytes from an untreated mouse were cultured for 48 h in medium containing 20% of that plasma or serum. The cells were then labeled with [^{35}S]methionine and the secreted proteins were analyzed as in Fig. 1. Besides the plasma proteins indicated with numbers (for identity see Fig. 1) two additional proteins, *a* and *b*, are detectable.

to have no detectable influence on the production of plasma proteins (see also below, Table III). Nevertheless, we have included lipopolysaccharides in the media of most control cultures to obtain proper conditions for comparison with cultures treated with monocyte media which also contains lipopolysaccharides.

In Fig. 3, we have shown the results of those treatments that yielded significant and reproducible changes in the secretion program. There are a number of features worth emphasizing. First, the comparison of the protein patterns of 0- and 48-h control demonstrates that the hepatocytes underwent a change in the production of secretory proteins. This property is expressed by a reduction in labeling of several proteins including albumin, α_1-antichymotrypsin, apolipoprotein A-I, and β-fibrinogen. At the same time, the production of two unidentified proteins is drastically increased (marked with *a* and *b* in Fig. 3). Second, when dexamethasone was present in the culture medium, both alterations seen in control cultures were prevented to some degree. The most pronounced effect of dexamethasone was the stabilization of α_1-antichymotrypsin production. Third, cells that had been treated with glucagon, like those exposed to mouse or rat serum (Fig. 2), specifically lost haptoglobin synthesis and secretion. Finally, simultaneous treatment of cells with glucagon and dexamethasone yielded a combined effect: preservation of the plasma protein synthesis, yet very low levels of haptoglobin (data not shown).

Conditioned medium from activated monocytes dramatically influenced the secretion program of the hepatocytes. All the major positive acute phase reactants: α_1-AGP, haptoglobin, hemopexin, and SAA were increased to a level comparable with that found in "inflamed" cells (see Fig. 1). The expression of some plasma proteins was maintained (e.g., α_1-antichymotrypsin), whereas others showed the changes seen in control cells (loss of β-fibrinogen and appearance of proteins *a* and *b*). Cells treated simultaneously with conditioned medium and dexamethasone developed unexpected changes. Most striking was the impairment of secondary modification of all glycoproteins, resulting in the release of undersialylated molecules into the medium. On the two-dimensional gel, these molecules are manifest as additional, more basic forms. This heterogeneity is comparable with that produced by neuraminidase digestion of normal plasma proteins (7). The identification of the series indicated on Fig. 3 has been demonstrated in the case of hepatoglobin and hemopexin by purification of the proteins and comparison with their normal counterparts (Fig. 4). This effect of conditioned monocyte medium and dexamethasone was observed in six independent preparations of hepatocytes and monocytes.

Quantitative Changes in Secretion of Plasma Proteins by Primary Hepatocytes

Separation of plasma proteins on two-dimensional gels as shown in Figs. 1–3 allows not only the qualitative and semiquantitative assessment of the secretion by hepatocytes but also a direct determination of [^{35}S]methionine incorporation into several protein species (Table I). Because the various treatments might affect uptake and intracellular pool of [^{35}S]-methionine differently, the measurements are expressed as values relative to total secretion. In this way, we can compare the secretory programs of the different cultures. The values obtained are not optimal because no correction for overall alteration in the secretion composition can be made (see Fig. 2). However, these measurements more accurately indicate the degree of modulation of plasma protein production than visual comparison of gel patterns. For instance, treatment with medium from activated monocytes leads to a fivefold increase in SAA and to a twofold increase in haptoglobin production by hepatocytes. Both changes lie in the range of those observed during an acute phase reaction. Also clearly illustrated is the stabilizing effect of dexamethasone on the production of α_1-antichymotrypsin.

A major criterion for selection of the proteins to be analyzed was their clear separation from other proteins. Accurate meas-

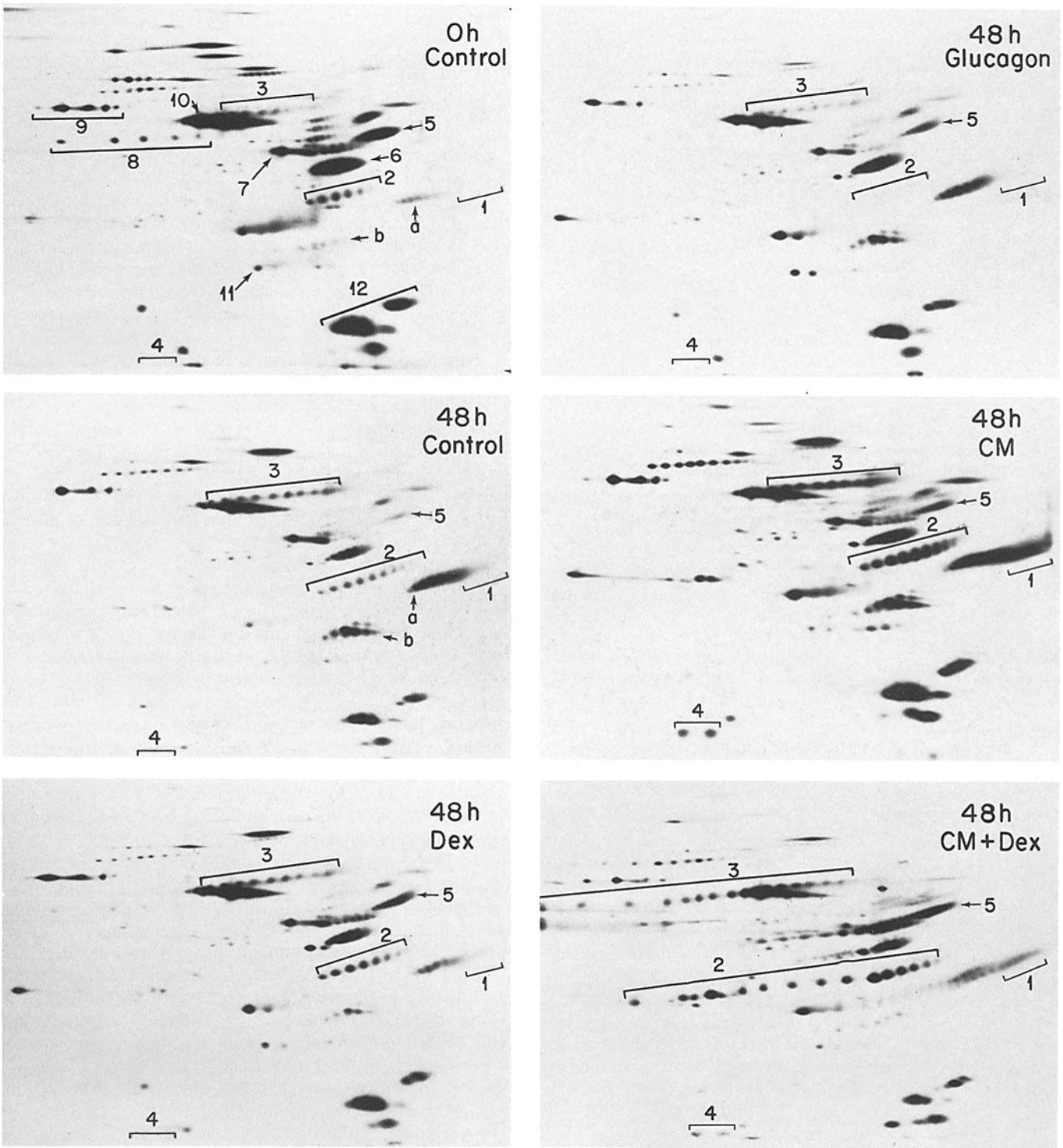

FIGURE 3 Effects of various culture conditions on the production of plasma proteins by hepatocytes. Hepatocytes from one individual mouse were plated into six-well plates (10-cm^2 culture surface) in culture medium containing 10% fetal calf serum and treated with 10 μg/ml lipopolysaccharide (*control*), 1 μM dexamethasone (*Dex*), 10 μM glucagon, conditioned monocyte medium (*CM*), and conditioned monocyte medium plus 1 μM dexamethasone (*CM + Dex*). At the times indicated, the cells were labeled for 6 h with [^{35}S]methionine. From each culture, 50 μl medium was analyzed by two-dimensional gel electrophoresis (see Fig. 1). The fluorographs were exposed for 24 h, except the gel *CM + Dex* which was exposed for 96 h. The proteins discussed in detail in text are: *1*, α_1-acid glycoprotein; *2*, β-haptoglobin; *3*, hemopexin; *4*, SSA; and *5*, α_1-antichymotrypsin.

urements of the radioactivity present in the spots for α_1-AGP, hemopexin, and albumin, were not possible because of the presence of interfering proteins (Fig. 2). Therefore, to obtain a quantitative estimate of changes in those proteins, we used different analytical techniques. α_1-AGP was purified from hepatocyte media by ion exchange chromatography followed by gel electrophoresis (Fig. 4). Haptoglobin was isolated by affinity chromatography on hemoglobin-sepharose columns and quantitated on polyacrylamide gels (Table II). Hemopexin, albumin, and haptoglobin were monitored by crossed

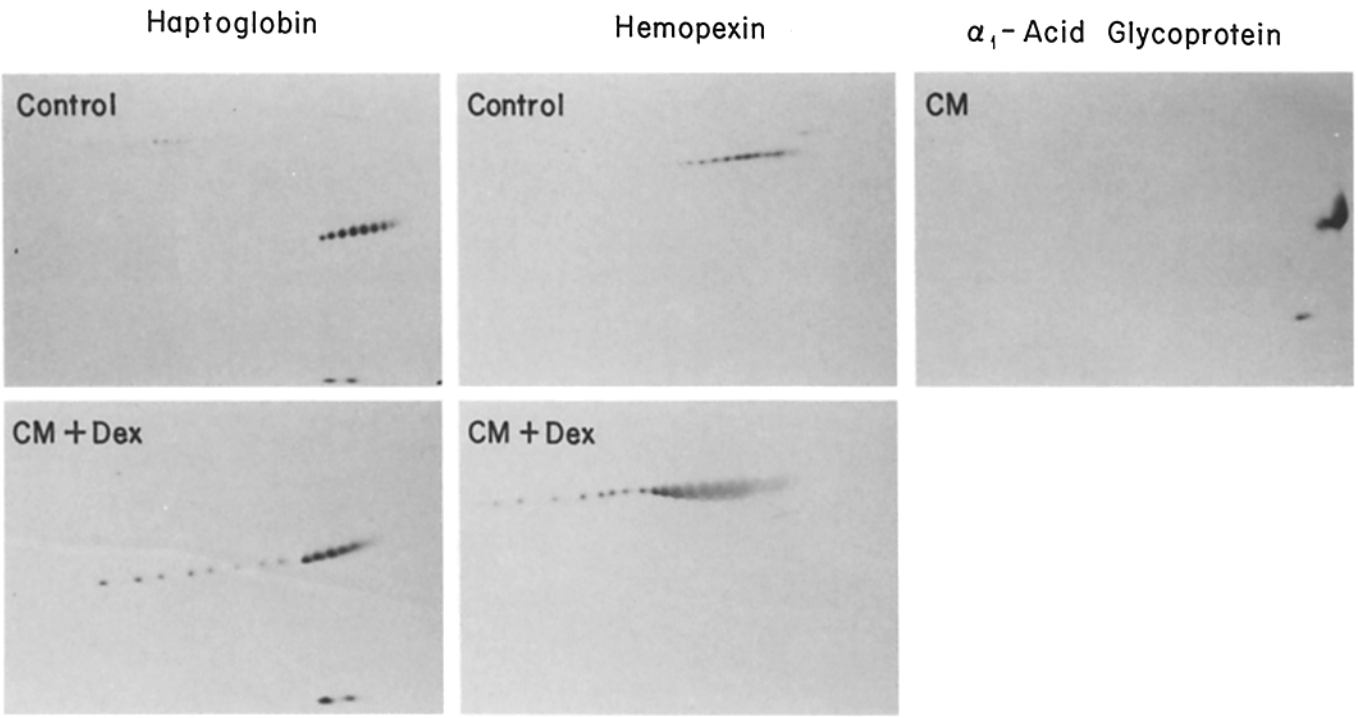

FIGURE 4 Electrophoretic properties of purified plasma proteins. From the media of hepatocytes that were cultured as in Fig. 3 for 48 h in Dulbecco's minimum essential medium (*control*), conditioned monocyte medium (*CM*), or conditioned monocyte medium plus 1 μM dexamethasone (*CM + Dex*) haptoglobin, hemopexin, or α_1-acid glycoprotein were purified. The isolated fractions were subjected to two-dimensional gel electrophoresis. For purification of hemopexin from cells in *CM + Dex*, we have included 200 μl of serum from a 24 h inflamed animal as carrier. The position of unlabeled serum hemopexin on the two-dimensional gel can be recognized by the distortion of the fluorographic image.

immunoelectrophoresis (Fig. 5 and Table III). Haptoglobin was measured in all analyses to obtain independently derived values for at least one major acute phase protein. The measurement of haptoglobin levels by three independent techniques; electrophoresis (Table I) hemoglobin binding (Table II), and immunoprecipitation (Table III) yielded essentially the same results. Only treatment with conditioned monocyte medium increased the synthesis and secretion of haptoglobin two- to threefold. Both α_1-AGP (Table II) and hemopexin (Table III) underwent a substantial increase in synthesis and secretion in control hepatocytes after 48 h in culture. Whereas dexamethasone had no further effect on these proteins, conditioned monocyte medium provoked an additional increase of the production of these two proteins.

The amount of albumin was regularly reduced by 50 to 75% during 48 h in tissue culture in all cases. Conditioned monocyte medium appeared to have the strongest inhibitory activity (Table III). Reduction in albumin production was noted during the acute phase.

Effects of Monocyte-derived Factors on Hepatocytes in Tissue Culture

The observation that all major hepatic acute phase reactants appear to be regulated in a similar fashion during an acute inflammation raises a question about the properties and action of the effectors. It is known that activated monocytes release factors including interleukin-1 (IL-1) and hepatocyte-stimulating factor, which have been found to modulate the synthesis and secretion of SAA and fibrinogen, respectively in primary hepatocyte cultures (24, 29). To assess the activity of these factors on the production of other acute phase proteins, we prepared a fraction from conditioned monocyte medium, which contains all components with molecular weights between 10,000–50,000, including IL-1 and hepatocyte-stimulating factor. Treatment of hepatocytes with this fraction resulted in the stimulation of α_1-AGP, hemopexin, and SAA, but not of haptoglobin (data not shown). The same pattern of modulation has been achieved by using conditioned monocyte medium from which components with molecular weight below 3,000 were removed by dialysis. This separation of modulating activity suggests that the acute phase proteins are regulated at least by two distinct factors released by activated monocytes.

Thus far, we have analyzed the biosynthetic response of primary hepatocytes by the patterns of secreted proteins. The question now arises as to whether these alterations in production of plasma proteins were a reflection of changes in the concentration of functional mRNA. To answer this, we extracted the RNA from control cultures and cultures treated with conditioned medium. The RNA were translated in a cell-free system and the products separated on two-dimensional gels (Fig. 6). This analysis reveals that the amount of functional mRNA for α_1-AGP, haptoglobin, and SAA were significantly enhanced in hepatocytes treated with monocyte factors. The fourth acute phase reactant, hemopexin, is not resolved in Fig. 6 owing to its basic charge (6). There are also many differences in the patterns produced by RNA prepared from intact livers and that from tissue culture cells.

Although it appears that the modulation of plasma protein synthesis was a result of a change in the level of functional mRNA, in at least one case post-translational modification also influenced the secretory phenotype. In Figs. 3 and 4, we have shown that the combination of conditioned monocyte

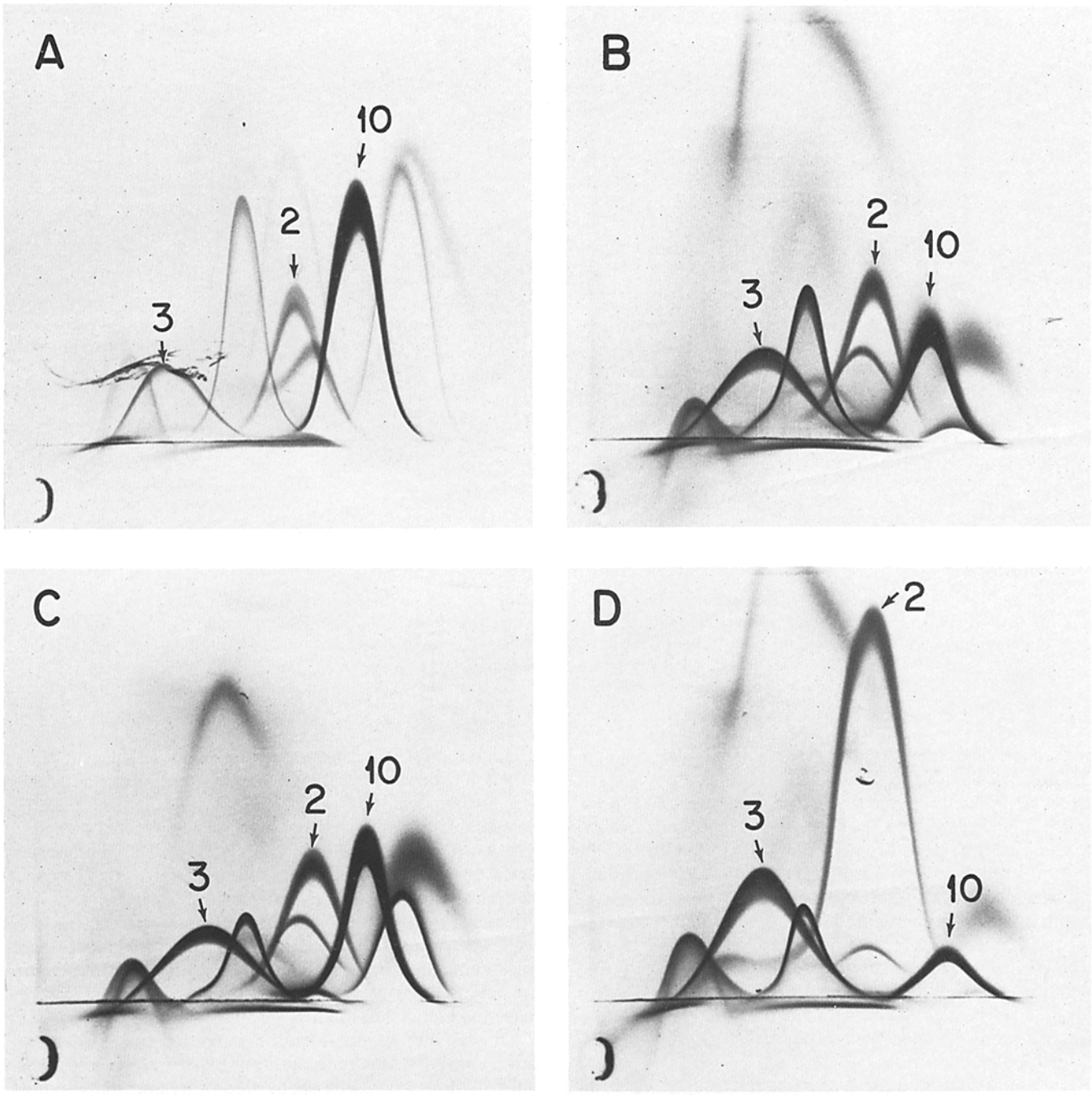

FIGURE 5 Crossed immunoelectrophoretic analysis of plasma proteins secreted by hepatocytes. Hepatocytes from one preparation were plated in 10-cm^2 dishes and were treated for 0 h (*A*) or for 48 h with lipopolysaccharide (*B*), lipopolysaccharide plus 1 μM dexamethasone (*C*), or conditioned monocyte medium (*D*). The cells were labeled with [^{35}S]methionine for 6 h. Secreted proteins present in 0.5 ml labeled culture medium were separated by crossed immunoelectrophoresis. The autoradiograms were exposed for 8 h. Numbers indicate the precipitin lines of: *2*, haptoglobin, *3*, hemopexin, and *10*, albumin.

medium and dexamethasone leads to release of aberrant plasma proteins. Measurements of isotope uptake and incorporation by the cells revealed that both were reduced as compared with control cells. More striking, however, was the finding that secretion was reduced 3- to 100-fold below control cells. We have consequently determined whether this reduction in secretion was accompanied by an intracellular accumulation of plasma proteins, using haptoglobin as an example. When we purified haptoglobin by affinity chromatography from control cell and cells treated with conditioned monocyte medium plus dexamethasone, we found that the latter cells contained an enormous intracellular accumulation of precursor forms (Fig. 7). Elution of this major β-chain precursor spot from a two-dimensional gel and digestion with endo-β-*N*-acetylglucosaminidase H in a third gel (8) revealed that almost all material was fully sensitive to the enzyme (data not shown). Quantitative measurements illustrate the extent of this intracellular accumulation (Table IV). We have included in these analyses hepatocytes treated with glucagon, to demonstrate that in those cells, the lack of haptoglobin production (see Fig. 3) was not due to a block in secretion but rather due to a specific reduction in synthesis. Cell-free trans-

TABLE I

Relative Synthesis and Secretion of Plasma Proteins by Hepatocytes

Cells	Medium Proteins (cpm/μg total cell protein)	Relative incorporation (× 10^4) SAA	β-Haptoglobin	α_1-anti-chymotrypsin	α_1-anti-trypsin	Anti-thrombin III	Transferrin
0 h control	18,300 ± 7,700	9 ± 5	142 ± 64	295 ± 102	848 ± 206	163 ± 54	190 ± 74
48 h LPS	10,000 ± 1,600	14 ± 5	157 ± 38	91 ± 38	646 ± 188	163 ± 43	252 ± 46
48 h LPS + Dex	15,300 ± 4,600	17 ± 4	192 ± 45	326 ± 49	647 ± 150	115 ± 26	173 ± 39
48 h CM	12,500 ± 2,900	65 ± 23	405 ± 103	122 ± 46	534 ± 119	114 ± 43	228 ± 71
0 h inflamed	17,300 ± 7,000	110 ± 40	228 ± 47	346 ± 86	570 ± 61	80 ± 20	77 ± 8

Primary hepatocytes were prepared from normal male mice and cultured for 48 h in medium containing 10 μg/ml lipopolysaccharide (*LPS*) or lipopolysaccharide and 1 μM dexamethasone (*LPS + Dex*), or in conditioned medium from activated monocytes (*CM*). These cells, as well as cells from mice 24 h after experimentally induced inflammation, were metabolically labeled with [^{35}S]methionine after the indicated times in culture. The secreted proteins were separated by two-dimensional gel electrophoresis as in Fig. 3. The radioactivity in the spots corresponding to the named plasma proteins were determined and expressed in values relative to the total amount of protein-bound radioactivity applied to the gels. Mean values and standard deviations from four to six independent cell preparations are shown.

TABLE II

Relative Synthesis of Haptoglobin and α_1-AGP

Hepatocytes	Relative incorporation (×10^4) Haptoglobin α	Haptoglobin β	α_1-AGP
0 h control	9	42	0.1
48 h LPS	8	41	4
48 h LPS + Dex	11	63	3
48 h CM	30	166	18
0 h inflamed	18	92	4

Hepatocytes were treated and labeled as in Table I. From the labeled culture media, 0.5 ml was removed and combined with 3,000 cpm of [^{3}H]fucose-labeled and chromatographically purified mouse α_1-AGP. Haptoglobin was quantitatively purified by chromatography of the media on immobilized hemoglobin (19). From the nonbound-fraction, α_1-AGP was isolated (15, 38). The haptoglobin fractions were separated into α and β subunits on a one dimensional 12% polyacrylamide gel. Each α_1-AGP fraction was separated by two-dimensional electrophoresis (see Fig. 4). The radioactivities in the gel-separated spots were determined and, in the case of α_1-AGP, corrected for loss during preparation using the recovery value of tritium. The measurements were expressed relative to the total protein-bound radioactivity in the initial medium fractions.

TABLE III

Synthesis of Albumin, Haptoglobin, and Hemopexin

Hepatocytes	Relative amounts of Albumin I	Albumin II	Haptoglobin I	Haptoglobin II	Hemopexin I	Hemopexin II
0 h control	1.00	1.00	1.00	1.00	1.00	1.00
48 h control	0.38	n.d.	0.92	n.d.	1.56	n.d.
48 h LPS	0.36	0.55	1.05	0.95	1.68	1.30
48 h LPS + Dex	0.44	0.55	1.17	1.46	1.52	1.22
48 h CM	0.22	0.19	2.86	2.42	2.69	1.90
0 h inflamed	0.27		2.45		2.52	

Hepatocytes (two separate preparations, I and II) were treated and labeled as in Table I. Aliquots of the labeled media (0.5 ml) were concentrated and separated by crossed immunoelectrophoresis (see Fig. 5). To quantitate the antigen amounts, the areas under the precipitin lines representing albumin, haptoglobin, and hemopexin were measured. The values were compared with the amount of total cell protein in each culture and then compared with the values of 0 h control cultures (defined as 1.00).

lation of RNA from glucagon-treated cells has already indicated that the amount of functional haptoglobin mRNA was much reduced relative to control cells (data not shown).

DISCUSSION

We chose primary cultures of mouse hepatocytes to study regulation of acute phase plasma protein synthesis. An attractive feature of mouse hepatocytes is that these are phenotypically consistent from one preparation to the other. The reason may be that the cells are derived from genetically inbred strains. The use of primary hepatocytes in tissue culture requires a compromise between the advantage that these cells offer the entire functional spectrum of fully differentiated adult liver cells and the disadvantage that the phenotype of these cells is not stable over time (23). Although such a culture system is not optimal, we were nevertheless successful in experimental modulation of the secretion program of the hepatocytes similar to that occurring in the liver during acute inflammation.

In vivo, the hepatic acute phase response was quite complex and includes a broad spectrum of manifestations. In this hepatocyte tissue culture system, however, expression is limited to four plasma proteins: α_1-AGP, haptoglobin, hemopexin, and SAA (Fig. 1). These four "positive" acute phase reactants served as principal markers in studying the response of hepatocytes. Although the liver is known to respond to a large variety of substances (33), we restricted our attention to hormones and other effectors that have been implied to regulate the hepatic acute phase reaction in vivo (22). The spectrum of effectors studied spans from a complex mixture, such as plasma, to single hormones. Hepatocytes, when exposed to plasma or conditioned monocyte medium, did show a simultaneous increased production of all four marker proteins (Tables I–III). The magnitude of induction for SAA is comparable with that reported by others (23, 26, 30). Ritchie et al. (29) employed a similar approach to modulate the plasma protein production of rat hepatocytes by monocyte-derived factors. They obtained a weaker stimulation of few antigenic plasma proteins. Surprisingly, haptoglobin was not one of them, although it represents a major acute phase protein in the rat (6). This discrepancy in activity might be due to the different sources of conditioned monocyte medium or different hepatocyte culture conditions.

In analysis of cells treated with conditioned monocyte medium, we showed that increased amounts of functional mRNA can account for the higher production of α_1-AGP, haptoglobin, and SAA (Fig. 6). Moreover, the same analysis demonstrated that the amount of functional mRNA for actin is not significantly affected by inflammation in vivo or by treatment of hepatocytes in tissue culture. When analyzing

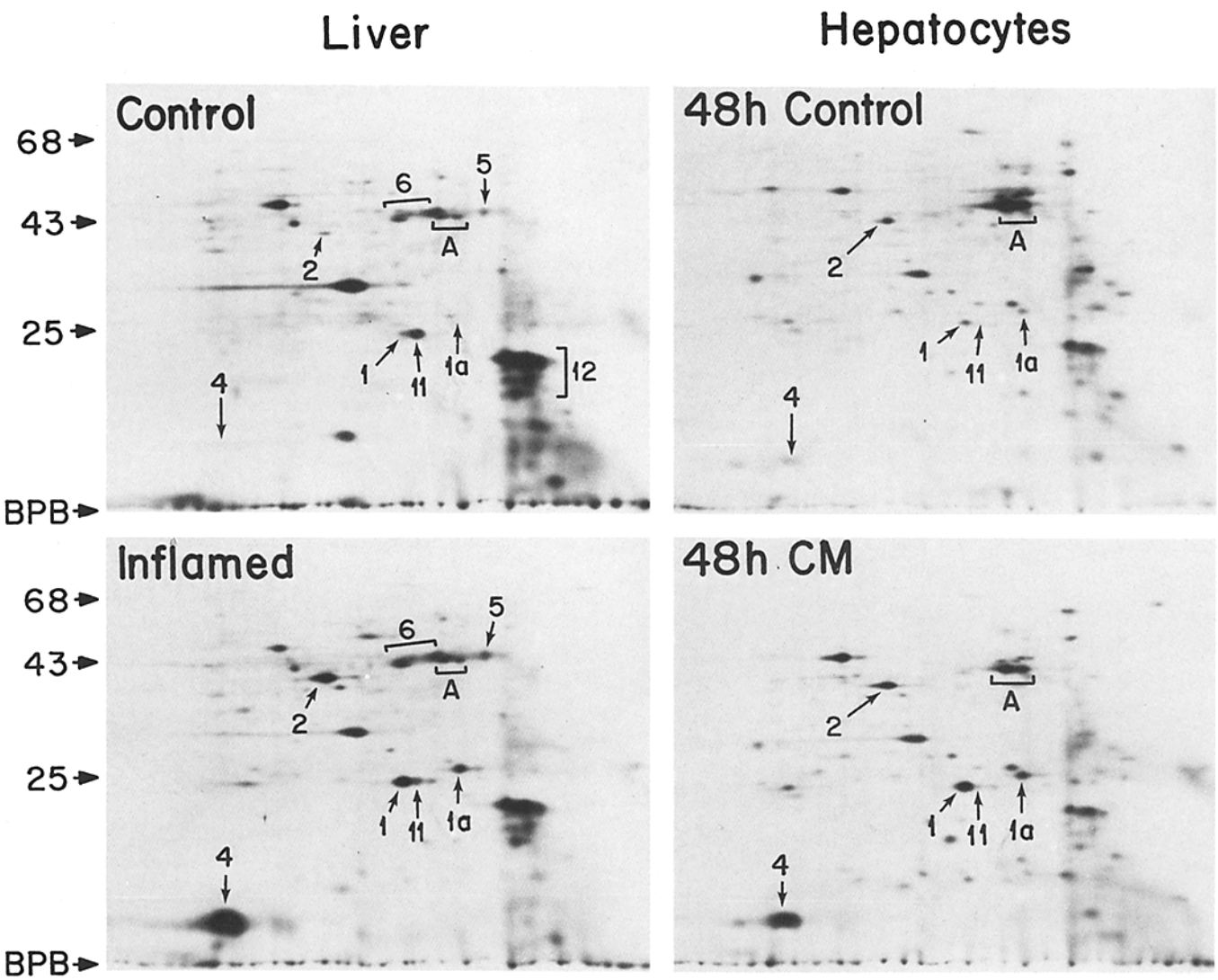

FIGURE 6 Modulation of functional mRNA in hepatocytes by monocyte-derived factors. Total RNA was extracted from hepatocytes, 1×10^7 cells in two 75-cm^2 flasks, which were either cultured for 48 h in Dulbecco's minimum essential medium (*control*) or in conditioned monocyte medium (*CM*). For comparison, RNA was similarly prepared from normal liver (*control*) or from a liver 24 h after a turpentine induced inflammation (*inflamed*). Aliquots of each RNA preparation, containing 5 μg RNA, were translated in a cell-free system and the products were separated by two-dimensional gel electrophoresis. The fluorograms were exposed for 3 d. The spots indicated by numbers represent precursor forms of: *1*, α_1-AGP; *1a*, α_1-AGP-like (this protein shares sequence homology with α_1-AGP; both proteins acquire 5-6 *N*-glycans and undergo processing to acidic plasma proteins; data not shown, Baumann, H., W. A. Held, and F. G. Berger, manuscript in preparation.); *2*, haptoglobin; *4*, SAA; *5*, α_1-antichymotrypsin; *6*, α_1-antitrypsin; *11*, apolipoprotein A-I; and *12*, major urinary proteins. *A* indicates spots corresponding to actin. *BPB*, bromphenol blue.

the effect 22 h after inflammatory stimulus on protein synthesis in mouse liver tissue, Morrow et al. (26) have, however, observed that actin undergoes a fivefold increase in synthesis and becomes the predominantly induced protein.

Although we have not carried out detailed characterization of the active components in conditioned monocyte medium, such as analysis of purified interleukin-1, the preliminary size fractionation of the components indicated the presence of more than one monocyte-derived effector. At this point, we cannot determine whether the haptoglobin-modulating activity is destroyed during dialysis and fractionation of the conditioned medium or whether the stimulation of haptoglobin synthesis is mediated by a small molecular weight component.

The addition of individual hormones to the culture media is a very restricted representation of in vivo conditions, but it is the best means to pinpoint activities contributing to the regulation of acute phase proteins. The result of dexamethasone treatment (Fig. 3) demonstrates that in the mouse, as opposed to the rat (6), none of the acute phase proteins were significantly affected. Dexamethasone was, however, effective in maintaining the phenotype of the hepatocytes, while suppressing the appearance of the two prominent "culture related" proteins. This hormone can be proposed as acting either by modulation of transcription rates or by influencing stability of mRNA.

Glucagon is a principal hormone involved in liver metabolism. One of its actions is also thought to be at the level of mRNA concentrations (14). The observation of this hormone's specific inhibition of haptoglobin synthesis was, however, unexpected. This action appears to be mediated via cAMP, for hepatocytes treated with 8-bromo-cAMP showed the same response (data not presented). In addition, mouse

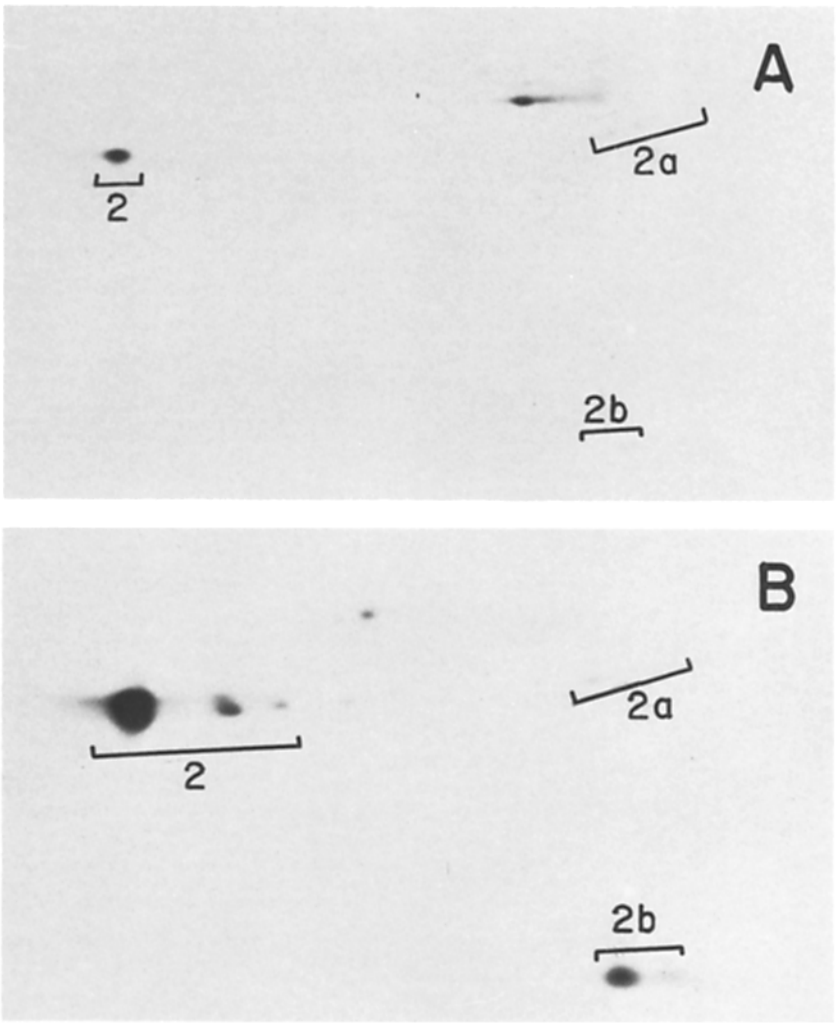

FIGURE 7 Intracellular form of haptoglobin. Haptoglobin was purified by affinity chromatography from soluble extracts of control cells (*A*) and cells treated with conditioned monocyte medium plus 0.1 μM dexamethasone (*B*) (for details see Table IV). The entire haptoglobin fractions were separated by two-dimensional gel electrophoresis. The fluorograms after 18 h exposure are shown. The spots indicated represent: *2*, cellular form of β-haptoglobin; *2a*, medium form of β-haptoglobin; and *2b*, α-haptoglobin. For quantitative measurements (Table IV), the most basic spot of the β-subunit (*2*) was used as representative of intracellular haptoglobin.

TABLE IV

Intra- and Extracellular Concentrations of Haptoglobin

Treatment	Amount of cell protein	Incorporation (CPM)			
		Cell protein	Cellular β-haptoglobin	Medium protein	Medium β-haptoglobin
	μg				
LPS	940	15.2×10^6	2,350	1.4×10^6	6,510
Glucagon	925	14.5×10^6	560	1.4×10^6	1,170
CM	745	12.7×10^6	7,490	1.1×10^6	21,720
CM + Dex	570	6.8×10^6	38,430	0.05×10^6	780

Monolayers of hepatocytes (10 cm^2) were cultured for 48 h in the presence of lipopolysaccharide (*LPS*), 10 μM glucagon, conditioned monocyte medium (*CM*), or conditioned monocyte medium plus 0.1 μM dexamethasone (CM + Dex). The cells were labeled for 6 h with 50 μCi/ml [^{35}S]methionine. The monolayers were washed three times with PBS and then homogenized by ultrasonication (5 s) in 1 ml of PBS containing 1 mM phenylmethylsulfonyl fluoride. Cell homogenates and media were centrifuged for 60 min at 200,000 *g*. Haptoglobin present in the supernatant fractions was affinity purified. Cellular haptoglobin was separated by two-dimensional gel electrophoresis (Fig. 7) and that from the media by one-dimensional gels. Radioactivity in β-haptoglobin only was determined. The values are expressed as radioactivity present in the entire fraction.

or rat sera specifically inhibited haptoglobin production (Fig. 2). We have already described this phenomenon in rat hepatocytes maintained in rat serum (6). It seems that in preparation of serum, an activity is generated during the coagulation process, which, although biochemically distinct from glucagon, might effect hepatocytes via a similar cellular mechanism.

The most intriguing result of our study was the metabolic response of hepatocytes to treatment with conditioned monocyte medium plus dexamethasone (Figs. 3 and 4). These cells showed a marked reduction in secretion of plasma proteins concomitant with intracellular accumulation of precursor forms (Table IV). The cause of the secretory impairment is not clear. The presence of early biosynthetic precursors intracellularly suggests that intracellular transport or processing of glycoproteins was affected, rather than the secretion process itself. The observation that secretion of nonglycosylated proteins, such as albumin and the major urinary proteins, was reduced proportionate to the glycoproteins, favors an impairment of intracellular transport over glycoprotein

processing. This inhibition of glycoprotein production is, however, different from that observed during glucose starvation. As we have recently reported (8), removal of glucose from culture medium of rat hepatoma cells leads to an immediate reduction in *N*-glycosylation of glycoproteins. Hepatocytes that were treated with monocyte factors and dexamethasone, still produced glycoproteins with a full set of *N*-glycans (see Figs. 3, 4, and 7).

The release of glycoproteins with partially complete carbohydrate moieties poses another problem to the hepatocyte. Some of these defective glycoproteins, which have not acquired a full set of sialic acid residues, are most likely displaying terminal galactose residues and would, therefore, be subject to endocytosis via receptors specific for galactose-terminated glycoproteins (18). Previous measurements have shown that mouse hepatocytes in tissue culture maintain the capacity for this specific receptor-mediated uptake of ligands for more than 48 h (K. C. Gaines and D. Doyle, manuscript in preparation). Because uptake and degradation of galactose-terminated glycoproteins takes place within minutes (11), recapture mechanism probably does not contribute significantly to this intracellular accumulation of plasma proteins.

We have demonstrated that individual hormones or factors that are known to be elevated during the acute phase, can modulate the production of plasma proteins in hepatocytes in tissue culture. These results provide insight into the complexity of the hepatic acute phase reaction. The question remains: how are these various factors involved and interrelated in vivo in the hepatic response? To unravel these regulatory events, one has to test varying concentrations and combinations of effectors. Furthermore, one must consider that the temporal sequence of various treatments might play an important part in programming mouse hepatocytes to properly execute an acute phase response.

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