Synthesis and secretion of C-reactive protein by rabbit primary hepatocyte cultures

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The synthesis and secretion of the acute-phase protein C-reactive protein by rabbit primary hepatocyte cultures was investigated. Hepatocytes prepared from animals that had received inflammatory stimuli 18–24 h before cell isolation were found to incorporate radiolabelled amino acids into C-reactive protein throughout the 48 h culture period. Intracellular C-reactive protein was found to be in steady state and there was no significant degradation of extracellular C-reactive protein, permitting direct estimation of rate of synthesis from rate of extracellular accumulation. Both C-reactive protein and total secreted protein were synthesized at constant rates for at least 24 h in culture. Mean rate of accumulation of newly synthesized total proteins in medium of cultures from six stimulated animals was 40% greater than was found in cultures from nine control (unstimulated) animals; this difference did not achieve statistical significance ($0.05 < P < 0.10$). Mean rate of C-reactive-protein synthesis represented 3.9% of total secreted-protein synthesis in cultures prepared from stimulated animals compared with 0.3% in cultures from control animals ($P < 0.001$). Further, there was a correlation between C-reactive-protein synthesis by cultured hepatocytes and serum C-reactive-protein concentration at time of hepatocyte isolation ($P < 0.001$). Rates of C-reactive-protein synthesis by hepatocyte cultures from stimulated animals were in good agreement with those previously measured in isolated perfused livers and those calculated from results of studies *in vivo*.

The acute-phase response is a complex systemic reaction to tissue injury or infection that includes an increase in serum concentration of a large number of proteins, the acute-phase proteins (Koj, 1974; Kushner, 1982). C-reactive protein (CRP), the first acute-phase protein to be identified, was initially described in man (Tillett & Francis, 1930). Recently, C-reactive proteins have been found in a number of phylogenetically diverse species (Baltz *et al.*, 1982; Robey & Liu, 1981). Although CRP is not a noteworthy acute-phase reactant in many of these species, it displays striking acute-phase charac-

teristics in both man and rabbit. The magnitude and kinetics of the CRP response are comparable in these two species, with acute-phase serum CRP concentrations rising rapidly and reaching values as high as 1000 times those found in normal subjects. In both species CRP is composed of five apparently identical polypeptide subunits non-covalently bound in cyclic symmetry (Gotschlich & Edelman, 1965; Osmand *et al.*, 1977*a,b*). The molecular weight of the rabbit protein is only slightly greater than that of the human protein (Oliveira *et al.*, 1980), and marked amino acid homology between these proteins has been shown (Oliveira *et al.*, 1979).

Serum CRP elevations in man parallel the course of the inflammatory response occurring in a variety of pathological conditions (Morley & Kushner, 1982; Pepys, 1981). Although details of the biological function of CRP are unclear at present, CRP exhibits a number of recognition and activation functions with the potential to modulate inflammation (Gewurz *et al.*, 1982). These properties may play a role in the local inflammatory response *in*

Abbreviations used: CRP, C-reactive protein; CxPS, Cx-polysaccharide [Cx-polysaccharide is extracted by the technique of Anderson & McCarty (1951). This is slightly different from the extraction of C-polysaccharide. Cx is the accepted term to distinguish these techniques.]; SDS, sodium dodecyl sulphate; Tris/saline, 0.01 M-Tris/0.15 M-NaCl/0.1% NaN₃, pH 7.4.

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vivo, as suggested by the observation that CRP accumulates in areas of necrosis within inflammatory lesions, but not in surrounding normal tissue (Kushner & Kaplan, 1961; DuClos *et al.*, 1981).

The site of CRP synthesis has been shown to be the liver (Hurlimann *et al.*, 1966) and specifically, the hepatocyte (Kushner & Feldmann, 1978). The increase in serum CRP levels seen during the course of the acute-phase response appears to be due to synthesis of CRP by increasing numbers of hepatocytes (Kushner & Feldmann, 1978). However, the mechanisms by which peripheral tissue injury leads to increased production of CRP are not clear. In order to further elucidate these mechanisms, we have developed methods to investigate the synthesis, secretion and stability of CRP in rabbit primary hepatocyte cultures. Rates of CRP synthesis by hepatocytes isolated from stimulated and unstimulated rabbits were determined and related to those previously observed in perfused rabbit livers and to rates calculated from experiments *in vivo*.

Materials and methods

Animals

Acute tissue injury was induced in New Zealand White rabbits weighing from 2 to 3 kg (obtained from Howard Gutman, Madison, OH, U.S.A.) by the intramuscular injection of 0.5–1 ml of turpentine into each thigh 18–24 h before preparation of hepatocytes. Control (unstimulated) rabbits received no inflammatory stimulus other than the surgery incident to hepatocyte isolation. Blood samples were obtained from the marginal ear vein before turpentine stimulation and at the time of hepatocyte preparation.

Cell cultures

Rabbit hepatocytes were prepared by a modification of the collagenase (Sigma type I) perfusion technique developed for rats by Laishes & Williams (1976). Freshly prepared hepatocytes were washed once in William's medium E (Gibco, Grand Island, NY, U.S.A.) and resuspended in the same medium containing $1\text{ }\mu\text{M}$ -cortisol. The percentage of viable cells (excluding 1.4% Trypan Blue dye) was determined with a haemocytometer. Foetal-calf serum (Gibco) was added to a final concentration of 10% (v/v), the concentration of viable cells was adjusted to 2×10^6 cells/5 ml, and 60 mm $\times$ 15 mm plastic culture dishes (Lux Scientific, Lab-Tek Division, Miles Laboratories, Naperville, IL, U.S.A.) were seeded with 5 ml portions. After a 1.5 h attachment period at 37°C in humidified air/CO₂ (19:1), the culture medium was replaced with 5 ml of serum-free William's medium E containing $1\text{ }\mu\text{M}$ -cortisol and the cells were incubated under the same conditions for the remainder of the culture period (usually 24–

48 h). All solutions for perfusion and cell culture contained 50 μg of tobramycin (Eli Lilly and Co., Indianapolis, IN, U.S.A.)/ml.

Initial attachment efficiency and cell survival throughout the culture period were determined from cell counts *in situ* on Trypan Blue-treated dishes. In later experiments, cells were allowed to attach in the absence of serum to culture dishes that had previously been coated with collagen (Sigma Type II). No differences in attachment, survival in culture or total protein synthesis were found when cultures of cells attached in the presence of serum were compared with those attached to collagen-coated dishes in the absence of serum.

Radiolabelling experiments

Unless otherwise stated, cells were incubated for variable periods in the presence of a ^3H -labelled L-amino acid mixture (NET-250; New England Nuclear Corp.) at a final concentration of 4 $\mu\text{Ci}/\text{ml}$. Radioactivity incorporated into total secreted protein was determined by trichloroacetic acid precipitation (final concn. 5%, w/v) of culture medium. Precipitates were collected by chimney-funnel suction on to GF/B glass-fibre filters (Whatman) and the filters were washed three times with 5 ml portions of 5% (w/v) trichloroacetic acid and once with 5 ml of acetone. Washed filters were placed in 10 ml of Scintiverse scintillation fluid (Fisher Scientific Co.) in glass vials and left at 4°C overnight before being counted for radioactivity in a Packard Tri-Carb scintillation counter with external standardization. Counting efficiency ranged from 20 to 30%. Determinations were corrected for background radioactivity measured in trichloroacetic acid precipitates of aliquots of culture medium to which the [^3H] amino acids had been added after harvesting the medium at the end of the culture period.

Detection of CRP

Radiolabelled CRP was isolated by affinity chromatography, taking advantage of the known Ca²⁺-dependent binding of rabbit CRP to CxPS (Anderson & McCarty, 1951). CxPS was extracted from a type II *Pneumococcus* (a gift from Dr. Emil Gotschlich) by the technique of Anderson & McCarty (1951), and was coupled to epoxy-activated Sepharose 6B (Pharmacia) by a modification of the procedure by Sundberg & Porath (1974). Portions (5 ml) of culture medium were passed over 1.5 ml of CxPS–Sepharose columns, and the columns washed with 30 ml of 0.15 M-Tris/0.05 M-CaCl₂/0.1% NaN₃, pH 7.0. Retained material was eluted with 0.15 M-sodium citrate/0.1% NaN₃, pH 7.0, and was found to be quantitatively precipitated in 5% trichloroacetic acid. After the addition of 100 μg of bovine serum albumin as carrier and 0.1 vol. of 50% (w/v) trichloroacetic

acid, the samples were left overnight at 4°C. The precipitates were collected on to filters, washed and counted for radioactivity as described above. Background radioactivity was determined by similarly processing an equal-sized portion of medium to which the [³H]amino acids had been added after harvesting the medium at the end of the culture period. Unlabelled CRP, present either in serum samples or in culture medium which had been concentrated 10–20-fold with Immersible CX filters (Millipore), was quantitatively detected by using radial immunodiffusion as described previously (Kushner & Somerville, 1971).

In later experiments, CRP present in culture medium was determined by using a sequential double-antibody radioimmunoassay. CRP (either as standards or in unknown samples) was incubated for 30 min at 37°C with an appropriate dilution of goat anti-(rabbit CRP) (usually 1:10⁶) in 0.85 ml of Tris/saline containing 0.1% bovine serum albumin. Purified ¹²⁵I-labelled CRP (see below) was added in an amount (usually 2 × 10⁵ c.p.m.) equal to twice the binding capacity of the antibody and the samples were incubated for 30 min at 37°C. The second antibody consisted of rabbit anti-(goat immunoglobulin G) covalently linked to nylon beads (Ab-SORB beads; Immuno-Reagents, Seguin, TX, U.S.A.); 5% Triton X-405/10 mM-EDTA (100 μl) containing 10 μl of beads was added and the samples incubated for 20 min at 25°C. After centrifugation at 5000 g for 20 min, the pellets were drained and counted for radioactivity in a Nuclear Chicago model 1085 gamma counter. The background tube contained no anti-CRP antibody and the maximum-binding (*B*₀) tube contained no unlabelled CRP. After subtracting background radioactivity, bound radioactivity (*B*) was expressed as a percentage of total radioactivity (%*B*) and was inserted into the following equation:

$$X = \text{logit} [(\%B/\%B_0) \times 100]$$

Y values for standards consisted of log[CRP]. Linear-regression analysis of the standard curve was used to compute unknown CRP concentrations from the observed percentage of radioactivity bound.

Preparation of radioiodinated CRP and CRP subunits

CRP was isolated from acute-phase serum obtained from rabbits, 24–48 h after intramuscular injection of 1 ml of turpentine, by affinity chromatography on CxPS–Sephadex. Purified CRP was iodinated by the lactoperoxidase method as described by Volanakis & Narkates (1981), with carrier-free Na¹²⁵I (New England Nuclear Corp.). Free Na¹²⁵I, ¹²⁵I-labelled lactoperoxidase and small amounts of aggregated ¹²⁵I-labelled CRP were removed by

CxPS–Sephadex chromatography followed by gel filtration on Sephadex G-200.

¹²⁵I-labelled CRP subunits were prepared by dialysis of purified ¹²⁵I-labelled CRP against 0.1 M-Tris, pH 9.0 for 36 h at 25°C. Undissociated CRP (usually less than 10% of total radioactivity) was removed by chromatography on Sephadex G-100 equilibrated in Tris/saline.

Detection of CRP subunits

The affinity-chromatographic method described above could not be employed for detection of radiolabelled CRP subunits, since CRP subunits were found not to bind to CxPS–Sephadex. We were able to demonstrate, however, that subunits were bound by our antiserum to rabbit CRP, as judged by a shift in elution volume of the ¹²⁵I-labelled subunits on gel filtration. Consequently, an immunoadsorption technique was developed for the detection of CRP subunits.

An IgG fraction was prepared from anti-CRP antiserum by DEAE-cellulose chromatography (Steinbuch & Audran, 1969). Approx. 3 mg of IgG was coupled to 1 g of CNBr-activated Sepharose 4B (Pharmacia). Immunoadsorption columns consisted of 1.5 ml of gel in 5 ml disposable pipettes. More than 70% of the radioactivity in ¹²⁵I-labelled CRP subunits was bound by such columns and resisted a wash with Tris/saline/6 M-urea. Bound radioactivity was eluted with 6 ml of wash buffer containing 0.1% SDS.

When this technique was employed to bind native CRP, elution resulted in dissociation of CRP into subunits. Thus, to distinguish subunits from pentameric CRP in unknown samples, it was necessary to fractionate samples on Sephadex G-100 before immunoadsorption. For analysis of the ³H-labelled culture medium, ¹²⁵I-labelled CRP and ¹²⁵I-labelled CRP subunits were added to samples to serve as markers of molecular size and percentage recovery. The Sephadex G-100 eluate was then divided into those fractions (*M*_r greater than 50 000) containing ¹²⁵I-labelled CRP and those (*M*_r less than 50 000) containing ¹²⁵I-labelled CRP subunits. Fractions thus identified were separately pooled and passed over anti-CRP–Sephadex columns. After the addition of *M*_r markers, the eluates were precipitated with 0.2 vol. of 50% (w/v) trichloroacetic acid. The 5000 g/10 min pellets were washed with acetone and subjected to SDS/polyacrylamide-gel electrophoresis on 12.5% gels (Laemmli, 1970). The gels were stained overnight in 0.5% Coomassie Blue, consecutive 1 mm sections were dissolved in 15% (v/v) H₂O₂, and ¹²⁵I radioactivity determined. Scintillation fluid (10 ml) was then added and the samples were stored overnight at 4°C. ³H radioactivity was corrected for the γ-radioactivity appearing in the ³H channel (usually 55–65%).

Extraction of intracellular proteins

Culture dishes were rinsed twice with Tris/saline and the cells in each dish were scraped in a total of 3–4 ml of Tris/saline/1% Triton-X 100/1% sodium deoxycholate. The resulting suspension was sonicated (model 185E Sonifier; Heat Systems–Ultrasonics, Plainview, NY, U.S.A.) with the small probe at 30 W for 2 min on ice. The 12 000 g/20 min supernatant was used for subsequent analyses. The extraction buffer did not affect chromatography on CxPS–Sephacrose or immunoadsorption columns. In the analysis of intracellular CRP by radioimmunoassay, the tubes comprising the standard curve contained appropriate volumes of extraction buffer.

Results

Cell cultures

The percentage of viable cells present in initial hepatocyte suspensions ranged from 70 to 94%. In 13 cultures from stimulated rabbits, $72 \pm 21\%$ (mean $\pm$ S.D.) of cells attached to culture dishes. In contrast, attachment efficiency was significantly less in 15 cultures from unstimulated rabbits: $54 \pm 22\%$ ($P < 0.05$). Cell survival during 48 h in culture did not differ in the two groups, averaging 93% in both groups at 24 h and 89% at 48 h. As described by others (Laishes & Williams, 1976), most cells assumed a polygonal shape within the first 24 h of culture life, and many were binucleate as they formed areas of confluent monolayers.

Quantification of CRP

Binding capacity of the CxPS–Sephacrose column for CRP was found to be more than 50 μ g when determined with purified radioiodinated rabbit CRP of known specific radioactivity. Recovery generally ranged from 90 to 95%. Radiochemical purity of the material present in the citrate eluate obtained from affinity chromatography of ^3H -labelled hepatocyte culture medium was examined in several ways. Gel filtration demonstrated that more than 95% of the radioactivity present in the citrate eluate emerged as a single symmetric peak corresponding to an apparent M_r of 105 000, consistent with that of rabbit CRP (Oliveira *et al.*, 1980). Analysis of radioactivity present in the citrate eluate by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) revealed a single band (Fig. 1) which migrated with an estimated M_r of 21 000, a value in good agreement with that of the rabbit CRP subunit (Oliveira *et al.*, 1980). In addition, more than 85% of the radioactivity in the eluate was precipitable with specific antibody to rabbit CRP.

The quantitative range of the radioimmunoassay for CRP was generally 0.25–25 ng of CRP/ml (final concn.). The use of sample volumes up to 100 μ l permitted a sensitivity of 2.5 ng of CRP/ml in

Fig. 1. Gel electrophoresis of CRP synthesized by isolated hepatocytes

Hepatocyte cultures (4×10^6 cells total) were incubated with ^3H -labelled amino acids ($4 \mu\text{Ci/ml}$) for 24 h and the culture medium was subjected to CxPS–Sephacrose chromatography as described in the Materials and methods section. After the addition of M_r markers and 5 vol. of 95% (v/v) ethanol, precipitated material was dissolved in SDS/polyacrylamide-gel electrophoresis sample buffer and analysed by electrophoresis on 10% (w/v) gels as described in the Materials and methods section. M_r markers (O) were identified as stained bands and represent: lysozyme (14 300), soya-bean trypsin inhibitor (21 000), carbonic anhydrase (30 000), ovalbumin (43 000) and bovine serum albumin (68 000).

unknown samples. The specificity of the radioimmunoassay for CRP was supported by the observation that standard curves generated with purified CRP and with culture medium containing CRP synthesized *in vitro* were parallel, straight lines (Fig. 2).

Synthesis, secretion and stability of CRP in hepatocyte cultures

The capacity of hepatocytes from stimulated rabbits to synthesize and secrete CRP throughout a 48 h culture period was assessed by measuring accumulation of radiolabelled CRP in culture medium during sequential 6 h incubations with ^3H -labelled amino acids. For the eight periods studied, mean incorporation of isotope into secreted CRP was 3220 ± 186 d.p.m./ 10^6 cells (mean $\pm$ S.E.M.).

The stability of extracellular CRP was determined by studying recovery of purified ^{125}I -labelled CRP which had been added to cultures after the attachment period. Medium was harvested at intervals and the amount of radioactivity remaining in pentameric CRP was determined by affinity chromatography on CxPS/Sephacrose. As shown in Table 1, there was no significant degradation of extracellular CRP during

Fig. 2. Radioimmunoassay for CRP

Known amounts of purified CRP were assayed by radioimmunoassay as described in the Materials and methods section (●). A portion of medium from a 24 h culture of cells prepared from a stimulated rabbit was concentrated 20-fold and the CRP concentration determined by radial immunodiffusion. The remainder of the unconcentrated culture medium was diluted with fresh Williams E medium to yield concentrations of CRP (○) approximating to those used in the standard curve prepared with purified CRP. Final concentrations of CRP in the assay ranged from 0.25 to 25 ng/ml in both cases. Slopes were determined by linear-regression analysis.

the first 24 h in culture, although substantial breakdown was noted by 48 h.

To investigate whether the observed accumulation of CRP in culture medium actually reflected active secretion rather than passive leakage through damaged cell membranes, we studied the effect of colchicine upon the intra- and extra-cellular distribution of ³H-labelled CRP. Colchicine, an inhibitor of active microtubule-dependent secretion, has been shown to inhibit CRP secretion *in vivo* (Kushner & Feldmann, 1978). Cells from a stimulated animal were preincubated for 2 h in 10 μM-colchicine and then incubated a further 6 h in the presence of ³H-labelled amino acids. Colchicine treatment resulted in a 40% decrease in secreted CRP and a concomitant 49% increase in intracellular CRP.

We studied the dynamics of the intracellular CRP pool by measuring the amount of CRP present in cells from a stimulated animal at intervals throughout the culture period. Intracellular CRP content was determined by radioimmunoassay of cell extracts from culture dishes, which were harvested at 2, 5, 8, 11 and 24 h after attachment. The amount of intracellular CRP was found to be relatively cons-

Table 1. Stability of extracellular CRP in hepatocyte cultures

Hepatocytes were prepared from a stimulated animal as described in the Materials and methods section. After the attachment period, the culture medium of replicate dishes was replaced with fresh medium containing purified ¹²⁵I-labelled CRP. At the indicated times, medium was harvested and stored at 4°C until chromatography on CxPS-Sephrose as described in the text. The control (zero time) sample of medium contained ¹²⁵I-labelled CRP, but was not incubated with hepatocytes before affinity chromatography. Data represent radioactivity present in the citrate eluates from each sample.

Time in culture (h)	10 ⁻³ × ¹²⁵ I-labelled CRP radioactivity (c.p.m.)	Recovery (%)
0	12.7	—
0.5	12.6	99.5
6	12.4	97.9
12	11.0	87.2
18	12.2	96.5
24	12.2	96.5
48	8.1	64.0

tant, ranging from 125 to 165 ng of CRP/10⁶ cells (mean = 148, S.E.M. = 6.7).

Since the intracellular pool of CRP appeared to be in steady state and there was only minimal, if any, extracellular degradation within the first 24 h of culture, the rate of CRP synthesis could be directly estimated from rate of accumulation in the medium. Accordingly, we employed the radioimmunoassay to measure the kinetics of accumulation of CRP in cultures prepared from stimulated animals. Both CRP (Fig. 3a) and total secreted protein (Fig. 3b) accumulated in the medium at linear rates for at least 24 h, indicating constant rates of synthesis during this period of time. Consequently, the accumulation of CRP in the medium over a 24 h period (whether determined by radioimmunoassay, radial immunodiffusion or ³H-labelled amino acid incorporation) was used as a measure of the rate of CRP synthesis in subsequent experiments.

Effect of stimulation *in vivo* on synthesis of CRP by hepatocyte cultures

As shown above, hepatocytes from stimulated animals synthesized readily detectable amounts of CRP. We compared rates of CRP and total protein synthesis by hepatocytes from six stimulated and nine unstimulated animals. As seen in Table 2, we were unable to demonstrate a statistically significant difference in total protein synthesis in the two groups. In contrast, cells from stimulated rabbits synthesized significantly more CRP than did cells prepared from control animals. Expressed as a

Fig. 3. Kinetics of accumulation of CRP and total protein synthesized by hepatocytes

Cells prepared from a stimulated animal were incubated in replicate cultures under standard conditions as described in the Materials and methods section. At the designated times medium was harvested and the viable cells were determined. CRP concentrations (a) were determined on duplicate 50 or 100 μ l portions by radioimmunoassay. Portions (3 ml) of medium were precipitated by the addition of 0.1 vol of 50% trichloroacetic acid and the 5000g/10min pellets were washed twice with 5 ml of 5% trichloroacetic acid. Total protein (b) was determined on washed precipitates by the procedure of Lowry *et al.* (1951), with bovine serum albumin as the standard.

percentage of total secreted-protein synthesis, the mean rate of relative CRP synthesis by cultures from stimulated rabbits was more than 10 times that of hepatocytes from control animals (Table 2).

This difference in accumulation of newly synthesized CRP in the medium did not appear to be due to decreased secretion of CRP by cells from unstimulated animals; the mean intracellular content of CRP (153 ng of CRP/ 10^6 cells) in four cultures from stimulated animals after 24 h in culture was

Table 2. Relative rate of CRP synthesis by hepatocyte cultures

Hepatocytes prepared from both stimulated and control animals were incubated for 24 h in the presence of ^3H -labelled amino acids. CRP and total protein radioactivity present in the medium were determined by affinity chromatography and trichloroacetic acid precipitation respectively. Details were as described in the Materials and methods section. Data are expressed as mean $\pm$ S.E.M., with the numbers of cultures in parentheses. The two groups were compared by the two-tailed Student's *t* test.

	$10^{-3} \times \text{Radioactivity}$ (d.p.m./24 h per 10^6 cells)		$\frac{(a)}{(b)} \times 100$
	(a) CRP	(b) Total protein	
Stimulated (6)	13.3 ± 3.59	454 ± 112	3.85
Control (9)	0.95 ± 0.19	323 ± 56	0.32
P-value	<0.005		<0.1, >0.05

more than eight times that (18.3 ng/ 10^6 cells) in two cultures from control animals. Further, the stability of CRP in culture medium from control animals was found to be similar to that in cultures from stimulated animals, indicating that the observed difference in accumulation of CRP was not due to differences in extracellular degradation.

In order to determine if the observed differences in rates of CRP secretion between cells from stimulated and control animals might be due to differences in rates of assembly of newly synthesized CRP subunits into CRP, we attempted to detect free CRP subunits. By using the immunoadsorption technique we were unable to detect CRP subunits in either intra- or extra-cellular compartments in cultures from either stimulated or control animals. The sensitivity of the method would have permitted detection of [^3H]CRP subunits representing 5% of the extracellular or 10% of the intracellular pools of CRP in the cultures prepared from stimulated animals. In the case of hepatocytes from control animals, radiolabelled subunits could have represented as much as 20% of the extracellular and 40% of the intracellular pools of CRP without being detected by this technique.

It is recognized that the serum CRP response to inflammatory stimulus may vary substantially from animal to animal (Yen-Watson & Kushner, 1974). In the present studies, serum CRP levels attained 20–24 h after intramuscular turpentine injection varied from 23 to 96 $\mu\text{g}/\text{ml}$ in 12 rabbits. The wide range of values for relative rates of CRP synthesis by cells from stimulated animals (Table 2) is consistent with this variability in CRP response to turpentine injection. Indeed, when rates of CRP synthesis by isolated hepatocytes were related to

Fig. 4. Correlation between CRP synthesis by hepatocytes and serum CRP concentration at time of hepatocyte isolation

CRP concentrations were determined by radial immunodiffusion of concentrated medium (●) or by radioimmunoassay of unconcentrated medium (O) obtained from 24 h cultures. Serum CRP levels were determined on samples obtained at the time of the animal's death. The data were examined by linear-regression analysis and the two-tailed Student's *t* test.

serum level attained at the time of hepatocyte isolation, a significant correlation was observed (Fig. 4).

Since synthetic rates observed *in vitro* may differ from those obtained *in vivo*, we compared rates of CRP synthesis determined in hepatocyte cultures with those previously found in a perfused liver system (Kushner *et al.*, 1980) and with those estimated from experiments *in vivo* in stimulated animals (Cheladurai *et al.*, 1982). For these purposes, findings were compared in three rabbits which were studied 20–24 h after inflammatory stimulus and which had achieved serum CRP levels ranging between 65 and 75 µg/ml by that time. Maximal synthetic rates *in vivo* were estimated from the observed half-life of intravenously injected ¹²⁵I-labelled CRP (Cheladurai *et al.*, 1982) and the steady-state serum CRP concentration. Assuming that there are 8.4×10^7 hepatocytes/g of liver (Laishes & Williams, 1976) and 40 g of liver per kg body weight (Webster *et al.*, 1947), rates of CRP synthesis were found to range between 0.42 and 0.56 mg/h per kg in all three cases.

Discussion

Newly synthesized CRP was shown to accumulate in the medium of primary hepatocyte cultures

prepared from both stimulated and unstimulated animals. CRP synthesized by hepatocytes was indistinguishable from that found in acute-phase rabbit serum, as judged by polymeric and subunit molecular sizes, antigenic reactivity, and Ca^{2+} -dependent binding to CxPS–Sepharose. By using immunoadsorption chromatography, we did not find evidence of secretion *in vitro* of larger or smaller molecules which might have represented precursors to CRP. This observation is in agreement with previous studies in which rabbit liver mince incubations did not produce detectable amounts of immunoprecipitable material of molecular size other than that of pentameric CRP (Kushner & Somerville-Volanakis, 1976).

We compared rates of synthesis and secretion of CRP by hepatocyte cultures from both stimulated and unstimulated rabbits. Since there was no detectable degradation of CRP present in culture medium for at least 24 h, the rate of secretion could be determined by measuring the rate of accumulation of CRP in culture medium. Further, since the intracellular pool of CRP appeared to be in steady state, the rate of CRP secretion was itself a direct reflection of the rate of CRP synthesis. We found that CRP was actively secreted by hepatocyte cultures and accumulated with linear kinetics for more than 24 h, with only a slight decline in rate by 48 h (Fig. 3). These observations indicate that CRP was synthesized by hepatocyte cultures at constant rates for at least 24 h.

Total protein synthesis was estimated by trichloroacetic acid precipitation of radiolabelled protein present in culture medium. Trichloroacetic acid precipitation was found to be suitable for our purposes; comparable results were obtained when we employed a different precipitation technique using phosphotungstic acid. The possibility that observed differences in CRP synthesis between hepatocyte cultures from stimulated and unstimulated rabbits might be subject to error due to differences in amino acid pool specific activities was obviated by expressing incorporation into CRP as a percentage of incorporation into total protein (Table 2).

We found a highly significant difference in CRP synthesis when we compared cultures from stimulated animals with cultures from control animals. Both intracellular CRP content and rate of CRP secretion were increased in cultures from stimulated rabbits. Previous studies have shown that tissue injury leads to continuing acceleration in hepatic CRP synthesis (Kushner *et al.*, 1980) and that progressively increasing numbers of hepatocytes can be shown to be synthesizing CRP during this period (Kushner & Feldmann, 1978). There was no evidence of acceleration in rate of CRP synthesis by hepatocyte cultures, suggesting that hepatocytes

which were actively synthesizing CRP at the time of cell isolation continued to do so *in vitro*, and that the apparent induction of CRP synthesis in additional cells seen *in vivo* requires extra-hepatic factors. This observation is in agreement with our previous findings in isolated perfused rabbit livers (Kushner *et al.*, 1980).

The average rate of total secreted protein accumulation in hepatocyte cultures from control animals was found to be $9.4 \pm 1.6 \mu\text{g/h}$ per 10^6 cells (results not shown). This is within the range of values previously reported for synthesis of total secreted protein by isolated hepatocytes (Feldhoff *et al.*, 1977) and is consistent with reports of albumin synthesis by hepatocytes (Jeejeebhoy *et al.*, 1976; Crane & Miller, 1974), assuming albumin to be 31–41% of total secreted-protein synthesis (Feldhoff *et al.*, 1977). Previous studies have described increases in synthesis of serum proteins during pneumococcal sepsis in rats (Powanda *et al.*, 1972) and an increase over control in secreted-protein synthesis by liver slices from turpentine-stimulated rats (Jamieson *et al.*, 1975). In our studies, rates of total secreted-protein synthesis by hepatocytes from stimulated animals were greater than those from controls, but we were unable to demonstrate a statistically significant difference (Table 2). It is possible that analysis of a larger number of experiments, including animals with a consistently marked acute-phase response would achieve statistical significance. In addition, many acute-phase proteins are glycosylated (Koj & Dubin, 1978). To the extent that such proteins might be inadequately precipitated by the acid-precipitation technique we employed, total protein synthesis might, in fact, be significantly greater in cultures from stimulated rabbits.

Concentrations of serum proteins are affected by rates of both synthesis and catabolism; the serum level of a protein may not be a direct reflection of its rate of synthesis (Koj & Regoeczi, 1978). However, several lines of evidence indicate that serum CRP concentration does, in fact, correlate directly with rate of synthesis. We have found that the fractional catabolic rate of CRP is constant, regardless of serum CRP concentration (Chelladurai *et al.*, 1982). Under such circumstances, increases in serum CRP levels would be expected to be determined primarily by increases in rate of synthesis. In the present studies, we found a good correlation between rate of CRP synthesis by hepatocyte cultures and serum level attained at time of the animal's death (Fig. 4). When we studied stimulated animals which manifested comparable CRP responses, we found good agreement between the rates of CRP synthesis determined in isolated hepatocytes and in perfused livers (Kushner *et al.*, 1980) and those estimated *in vivo* (Chelladurai *et al.*, 1982).

Serum amyloid P component, a plasma protein with structural homologies with CRP (Osmand *et al.*, 1977a), exhibits Ca^{2+} -dependent binding to Sepharose (Pepys *et al.*, 1977) and thus might be expected to contaminate CRP in the citrate eluate of our affinity-chromatographic columns. However, the M_r of rabbit serum P component has been reported to be nearly identical with that of the human protein, estimated at 255 000 (Pepys *et al.*, 1977). Thus radiolabelled P component present in our citrate eluate would have been readily distinguished from CRP by filtration on Sephadex G-200, where it would have appeared in the void volume. Significant amounts of radioactivity were not observed in the void volumes of such columns. We conclude that newly synthesized P component, which may have been present in the CxPS-Sepharose citrate eluates, did not contribute significant amounts of radioactivity.

The observation that cells isolated from stimulated animals attached to culture dishes with greater efficiency than did those from controls is noteworthy. The mechanisms responsible for this difference are not clear, but may be related to the recently described role of intracellular actin in cellular attachment (Singer, 1979). Studies in a cell-free system utilizing mRNA derived from mouse liver have shown that tissue injury results in marked induction of actin synthesis (Morrow *et al.*, 1981).

Two recent studies have reported that synthesis of several acute-phase proteins by primary hepatocyte cultures prepared from stimulated rats was increased compared with cultures from unstimulated animals (Howlett *et al.*, 1981; Fouad *et al.*, 1980). In both studies, albumin synthesis and secretion by control cells and cells from stimulated rats appeared to be similar.

The mechanisms by which tissue injury leads to increased hepatic synthesis of CRP and of the other acute-phase proteins are unclear. There is evidence that different mechanisms may be responsible for the responses of different acute-phase proteins (Sipe *et al.*, 1982). Several studies *in vivo* have suggested that the cytokine Interleukin 1 may play a key role in the mediation of many acute-phase phenomena (Sztein *et al.*, 1981; Bornstein & Walsh, 1978). Interleukin 1 has recently been reported to cause moderate increases in synthesis of haptoglobin *in vitro* (Hooper *et al.*, 1981) and fibrinogen (Rupp & Fuller, 1979) by rat hepatocytes, and of serum amyloid A protein by mouse hepatocytes (McAdam *et al.*, 1982). Induction of CRP synthesis *in vitro* has not been shown. The hepatocyte culture system described here appears suitable for investigation of the molecular mechanisms which regulate CRP biosynthesis.

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