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The effects of 17α-ethynyloestradiol and of acute inflammation on the plasma concentration of rat α_1-acid glycoprotein and on the induction of its hepatic mRNA

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We measured the serum concentration of α_1-acid glycoprotein (α_1-AGP) and we evaluated the content of its hepatic mRNA in rats after 17α-ethynyloestradiol treatment or after turpentine-induced acute inflammation, or after both treatments performed simultaneously. We have also studied the affinity of serum α_1-AGP for concanavalin A under these conditions. Both types of stimuli induce a marked retention of the glycoprotein on free concanavalin A. The serum concentration of α_1-AGP is increased about 14-fold compared with that in control rats when a single pharmacological dose (50μg) or multiple injections of 17α-ethynyloestradiol are administered. This increase is greater in turpentine-oil-injected rats (about 21-fold) and reaches a maximum (about 32-fold) in rats injected with 17α-ethynyloestradiol plus turpentine oil; this increase in α_1-AGP corresponds to the addition of the effects of the two inducing agents. Similar changes are also observed either in the α_1-AGP mRNA content as estimated by using an α_1-AGP-specific cDNA probe, or in the amount of translatable α_1-AGP mRNA. The results indicate that: (1) after a high dose of 17α-ethynyloestradiol and after acute inflammation, the increase of the α_1-AGP serum concentration is due to an accumulation of the α_1-AGP mRNA; (2) different mechanisms and/or pathways are probably involved in regulating the synthesis of α_1-AGP under various stimuli; (3) 17α-ethynyloestradiol as well as acute inflammation seem to control the glycosylation process of α_1-AGP in an identical manner.

The biosynthesis in the liver of many plasma proteins is oestrogen-sensitive (Barbosa *et al.*, 1973). Pregnancy and treatment with exogenous oestrogens have been found to influence the hepatic biosynthetic rate of plasma proteins in opposite directions (Song *et al.*, 1970; Barbosa *et al.*, 1973; Laurell & Rannevik, 1979). An increase in hepatic synthesis of plasma renin substrate in the rat (Eisenfeld *et al.*, 1977), as well as increases in mRNA sequences for lipoprotein and vitellogenin in avian liver (Deeley & Goldberger, 1979; Chan *et al.*, 1979), have been directly correlated with the presence of oestrogens.

Abbreviations used: α_1-AGP, α_1-acid glycoprotein; SDS, sodium dodecyl sulphate.

The increase in blood concentration of a large number of liver-produced plasma proteins, the so-called acute-phase proteins (Koj, 1974), results from tissue injury, but the mechanisms whereby the acute inflammation leads to increased hepatic synthesis by the liver during acute inflammation is thought to be mediated by multiple effector systems (Koj, 1974). A part of acute-phase response seems to be glucocorticoid-mediated, since some acute-phase reactants are stimulated by adrenalectomy (Gordon & Koj, 1968), whereas others are unaffected. Several studies (Baumann & Held, 1981; Baumann *et al.*, 1983) indicated that glucocorticoid treatment of rat hepatoma cells or turpentine-induced inflammation in rats both induce an increase in rat α_1-AGP mRNA. The aim

of the present study was to establish whether oestrogens could play a role in modulating the hepatic synthesis of the acute-phase reactant α_1-AGP, and to determine the mechanism of this modulation.

To study this problem, we have measured the serum concentration of α_1-AGP and we have quantified its hepatic mRNA in rats after 17α-ethynyloestradiol treatment or after turpentine-induced acute inflammation, or both treatments performed simultaneously.

The results demonstrate that induction of α_1-AGP serum concentration by 17α-ethynyloestradiol and acute inflammation is due to an accumulation of the α_1-AGP mRNA, and that different mechanisms are probably involved in regulating the synthesis of α_1-AGP.

Materials and methods

Reagents

Materials were obtained from the following sources: 17α-ethynyloestradiol, yeast tRNA type VI, ATP, GTP, dithiothreitol, haemin and spermidine, from Sigma; Tris, sucrose, Triton, phenol, chloroform and unlabelled amino acids from Merck; phosphocreatine from Serva; CM-Trisacryl R_M, concanavalin A and Ultrogel AcA-44 column, from I.B.F. (France); heparin from Laboratoire Leo; protein-A–Sepharose from Pharmacia; oligo(dT)–cellulose chromatography column from Collaborative Research; [^{35}S]-methionine (1160Ci/mmol), ^{14}C-labelled M_r markers, Aquasol and [^{32}P]dXTP (3000Ci/mmol) from Amersham; restriction endonuclease *PstI* from BRL; all other reagents were A.R. grade, where available.

Experimental procedure

Acute inflammation was induced in Sprague–Dawley male rats (250–350g) (IFFA-CREDO, France) by a single subcutaneous injection of turpentine oil (0.6ml/100g body wt.) in the dorsal lumbar region. At 48h after injection, the rats were anaesthetized with diethyl ether, and killed by decapitation. Blood thus obtained was allowed to clot for 12h at 4°C. Serum was obtained after centrifugation at 1000*g* for 15min. The livers were excised and rapidly frozen in liquid N_2. Hormone treatment of male rats was performed by a subcutaneous injection 0.2ml of sesame oil containing 50μg of 17α-ethynyloestradiol. Serum and livers were obtained as described above. In kinetic experiments, small samples of blood were collected from the tails at suitable times.

Protein purification and antibody production

α_1-AGP was extracted from serum obtained 48h after turpentine injection and was purified essentially as described by Nicollet *et al.* (1981) for human α_1-AGP. The homogeneity of the protein was established by SDS/polyacrylamide-gel electrophoresis and by immunoelectrophoresis using antisera against α_1-AGP and against the normal rat serum. Antisera to α_1-AGP and to normal rat serum were raised in rabbits by injecting 500μg of α_1-AGP emulsified with an equal volume of complete Freund's adjuvant into the hind footpads. Booster amounts were given subcutaneously until satisfactory titres were obtained.

Quantification of serum α_1-AGP, and immunoelectrophoretic techniques

The concentration of serum α_1-AGP was determined by single radial immunodiffusion by using monospecific α_1-AGP antiserum and purified rat α_1-AGP as the standard (range 0.66–0.12g/l), whose mass had been determined by drying to constant weight. Immunoelectrophoresis was carried out in 1.3% agar in 0.05M-barbital buffer, pH8.6. Crossed immuno-affino-electrophoresis was performed as described previously (Bøg-Hansen *et al.*, 1975; Nicollet *et al.*, 1981) with or without concanavalin A in the first dimension. The second dimension was performed in a 1%-agarose gel, 1mm thick, containing anti-(α_1-AGP) antiserum (50μl/7.5ml) and 0.2M-methyl glucopyranoside (Salier *et al.*, 1980, Raynes, 1982). Peak areas of the protein patterns were measured by planimetry.

Preparation of poly(A)-containing RNA

Total RNA was extracted from frozen liver. The tissue was first pulverized in liquid N_2 in a Waring Blendor and then homogenized in a tissue grinder (Duall Kontes) with 3ml of Tris buffer (200mM-Tris/HCl, pH8.6, 25mM-$MgCl_2$, 50mM-KCl, 200mM-sucrose, 500μg of heparin/ml, 4mg of yeast tRNA/ml)/g of tissue and then adjusted to 1% deoxycholate and 1% Triton X-100. The homogenate was centrifuged at 5000rev./min in a Beckman SW 27 rotor for 15min at 4°C. The supernatant was diluted (1:1, v/v) with 0.1M-sodium acetate buffer (pH5)/1% SDS, and RNA was purified by two successive oligo(dT)–cellulose chromatographies of ethanol-concentrated RNA (Bantle & Hahn, 1976).

Cell-free translation and immunoprecipitation

Poly(A)-containing mRNA was translated in a mRNA-dependent rabbit reticulocyte lysate as

described by Pelham & Jackson (1976). The translation of rat mRNA in the presence of [^{35}S]-methionine (1160Ci/mmol) and radioactivity counting of the trichloroacetic acid-precipitable translation products have been previously detailed (Vercaigne *et al.*, 1982).

Immunoprecipitation was performed essentially as described by Vercaigne *et al.* (1982). Briefly, translation mixture (5μl) was mixed with 5μl of anti-(rat α_1-AGP) antiserum supplemented or not with 10μl of unlabelled α_1-AGP (1, 10 or 100μg). Then 30μl of protein-A–Sepharose were added and the mixture was incubated overnight at 4°C with continuous shaking. The protein-A-bound immune complexes were eluted and electrophoresed. For quantification, immunoprecipitable radioactivity was measured in 5μl samples. Translated and immunoprecipitated products were analysed by electrophoresis on vertical polyacrylamide gels containing SDS (Laemmli, 1970). Gels were treated for fluorography (Bonner & Laskey, 1974). Autoradiographs were exposed for 12–36h at −80°C on Kodak Blue Brand Regulix films. Densitometric quantification of polypeptide bands was performed on a Gelman DCE apparatus.

RNA blot hybridization analysis

Poly(A)-containing mRNA (1.2 and 4μg) was denatured at 50°C with aq. 50% (v/v) dimethyl sulphoxide containing 1M-glyoxal and examined by electrophoresis in 1.5%-agarose gels (McMaster & Carmichael, 1977). The RNA was transferred to nitrocellulose (Thomas, 1980), and hybridized to cDNA insert from plasmid pAGP663 (Ricca *et al.*, 1981). The [^{32}P]cDNA probe for this hybridization was prepared as follows: 75μg of plasmid DNA from the clone pAGP663, which contains the entire coding region for α_1-AGP, was digested with the restriction endonuclease *PstI*. The 740-base-pair *PstI* fragment was isolated by preparative 6%-polyacrylamide-gel electrophoresis and labelled by nick-translation (Rigby *et al.*, 1977) to a specific radioactivity of about 10^8c.p.m./g. Buffers and conditions for prehybridization and hybridization were those described by Wahl *et al.* (1979). Fluorography of nitrocellulose filters and densitometric quantification of the bands were performed as above.

Results

Kinetics of changes in serum α_1-AGP concentration after hormonal treatment and experimental inflammation

High doses of 17α-ethynyloestradiol have been shown to effect maximal translocation of the cytosol oestrogen receptors to the nucleus (Marr *et al.*, 1980*a*). In our study, we administered doses (50μg) 5 times those used by Marr *et al.* (1980*b*). Serum α_1-AGP concentration was evaluated for 7 days (Fig. 1) after 17α-ethynyloestradiol administration (first group) or turpentine-oil injection (second group). A maximum value was obtained at 2 days: 1.81 ± 0.46mg/ml (mean ± 1S.D.) for the first group and 2.71 ± 0.71mg/ml for the second group which represent respectively about 14-fold and 21-fold increases from the physiological value. A dose of 17α-ethynyloestradiol administered at day 2 and day 4 to the rats of the first group did not significantly alter the increase in serum concentration obtained after a single injection. When the rats of the first group also received an injection of turpentine oil alone (third group) or together with 17α-ethynyloestradiol (fourth group) at day 2, the serum α_1-AGP increased drastically at day 3, to 4.12 ± 0.52mg/ml (about 32-fold). This dramatic increase observed when a maximal effect of oestrogen is obtained corresponds to the sum of the effects of the two inducing agents. At day 4, the difference in α_1-AGP concentration observed between the third and the fourth groups corresponds approximately to the effect of the additional injection of 17α-ethynyloestradiol at day 2.

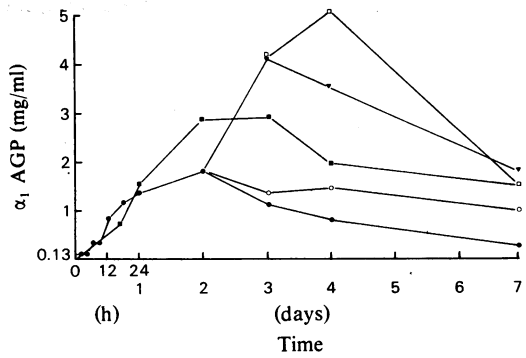

Fig. 1. *Kinetics of change in serum α_1-AGP concentration after 17α-ethynyloestradiol treatment and turpentine-oil-induced inflammation*

The serum α_1-AGP concentration was determined by single radial immunodiffusion. ●, Rats injected with 50μg of 17α-ethynyloestradiol (first group). ■, Rats injected with turpentine oil. ○, First group of rats injected again with 17α-ethynyloestradiol at days 2 and 4. ▼, First group of rats injected again with turpentine oil at day 2. □, First group of rats injected again with turpentine oil and 17α-ethynyloestradiol at day 2. Each point represents the mean value of determinations in five rats.

Changes induced by 17α-ethynyloestradiol and acute inflammation in the immunoelectrophoretic pattern of rat serum α_1-AGP

When crossed immuno-affino-electrophoresis with concanavalin A was performed with methyl

α-D-glucopyranoside, four α_1-AGP peaks could be distinguished in the sera of normal rats (Fig. 2*b*): respectively a component reactive with concanavalin A (peak 1), a component (peaks 2 and 3) weakly reactive with concanavalin A and a component not reactive with concanavalin A (peak 4). Crossed-immuno-affino-electrophoresis patterns of sera from normal rats and of sera from rats 48h after administration of 17α-ethynyloestradiol or of turpentine oil were quantified (Table 1). Both 17α-ethynyloestradiol and turpentine-oil administration principally increased the amounts of the components reactive and weakly reactive with concanavalin A.

Synthesis in vitro of α_1-AGP after hormonal treatment and after acute inflammation

Since there was an increase in the serum concentration of α_1-AGP, reaching a maximum at 48h after administration of 17α-ethynyloestradiol or turpentine oil, or after simultaneous injection of these two agents, we have chosen this time for preparing total rat liver poly(A)-containing mRNA from the treated animals. We first verified that the messenger activity of mRNA preparations from either group in the same experimental series was the same or nearly the same. The method, standardized as described previously (Pelham & Jackson, 1976; Vercaigne *et al.*, 1982), was highly reproducible in terms of maximal translational activity and RNA dose-dependence. The difference in the messenger response between two RNA preparations never exceeded 10%, and the efficiency of translation of poly(A)-containing mRNA of treated and control rats was identical (Table 2). Fig. 3 shows the autoradiographs of the cell-free translation products of poly(A)-containing mRNA from control and treated rats and ^{35}S-labelled poly-

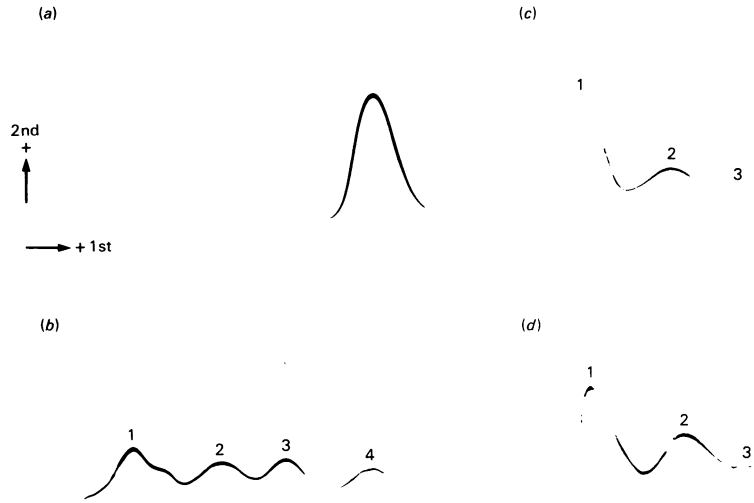

Fig. 2. *Crossed affino-immuno-electrophoresis with concanavalin A of normal rat serum and of rats injected with either 17α-ethynyloestradiol or turpentine oil*

Samples used: (*a*) and (*b*), 15μl of normal rat serum; (*c*) 15μl of serum from 17α-ethynyloestradiol-treated rat diluted 1:1 in 0.15M-NaCl; (*d*) 15μl of serum from turpentine-injected rat, diluted 1:5 in 0.15M-NaCl. Concanavalin A (1.5mg/ml) was included in the first dimension (*b*, *c*, *d*), but electrophoresis was run without it in (*a*). Peaks are identified by arbitrary numbers (1–4): peak 1 corresponds to the highest affinity for concanavalin A.

Table 1. *Relative proportions of microheterogeneous components of rat serum α_1-AGP as determined after crossed immuno-affino-electrophoresis with concanavalin A in the first dimension*

Peaks are expressed as a percentage (±S.D.) of the total protein profile area (see Fig. 2).

	Percentage of α_1-AGP precipitate area			
	Peak 1	Peak 2	Peak 3	Peak 4
Untreated ($n = 10$)	30±4.5	28±2.8	24±2.5	18±3
17α-Ethynyloestradiol-treated rats ($n = 5$)	55±2	30±1	13±1	2±0.5
Turpentine-injected rats ($n = 6$)	45±4	44±3	11±2	

peptides immunoprecipitated with antiserum to α_1-AGP. Though the translation of mRNA was carried out in identical conditions and the same amount of radioactivity was used for immunoprecipitation, it is evident that treatment of rats *in vivo* with 17α-ethynyloestradiol or turpentine oil, or a combination of both, increased the amount of a peptide of apparent M_r 23000 (Fig. 3). When increasing amounts of unlabelled rat α_1-AGP were added to the translation mixture, inhibition of immunoprecipitation was obtained, which confirmed that the immunoprecipitated product was α-acid glycoprotein.

The α_1-AGP specific radioactivity as determined after densitometric analysis increased strongly (11-fold) in 17α-ethynyloestradiol-treated rats compared with that observed in control rats. This activity was greater in turpentine-oil-injected rats (28-fold) and reached a maximum (40-fold) in rats treated with the two inducing agents together (Table 3).

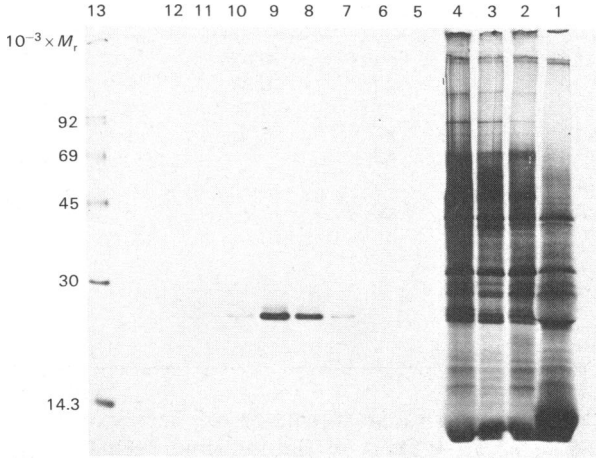

Fig. 3. *Cell-free synthesis of rat α_1-AGP*
Total rat liver poly(A)-containing RNA (0.4 μg) was translated and analysed by electrophoresis on 15% polyacrylamide gels in sodium dodecyl sulphate before (1–5) and after immunoprecipitation with anti(α_1-AGP) (6–12). Tracks: 1, 6, mRNA from unstimulated rats; 2, 7, mRNA from 17α-ethynyloestradiol-treated rats; 3, 8, mRNA from turpentine-oil-treated rats; 4, 9, mRNA from rats simultaneously treated with two inducing agents; 10, 11, 12, as tracks 4 and 9, except that the anti-(α_1-AGP) antiserum was previously incubated with purified protein (10, 1 μg; 11, 10 μg; 12, 100 μg); 5, without exogenous mRNA; 13, M_r markers: lysozyme (14300), carbonic anhydrase (30000), ovalbumin (45000), bovine serum albumin (69000), phosphorylase *b* (92000).

Quantification of α_1-AGP mRNA

Poly(A)-containing mRNA was prepared from livers of control rats and from rats at 48h after treatment. The results of mRNA blot hybridization analysis indicated that α_1-AGP mRNA was approx. 850 bases in length, in livers of control and treated rats (Fig. 4). There was about a 9-fold, 36-fold and 46-fold increase in α_1-AGP mRNA in 17α-ethynyloestradiol-, turpentine-oil- and simultaneously hormone-treated and inflamed rats respectively, compared with that observed in control rats. These values are elevated to the same extent as those observed for the primary translation products of α_1-AGP mRNA. However, these values

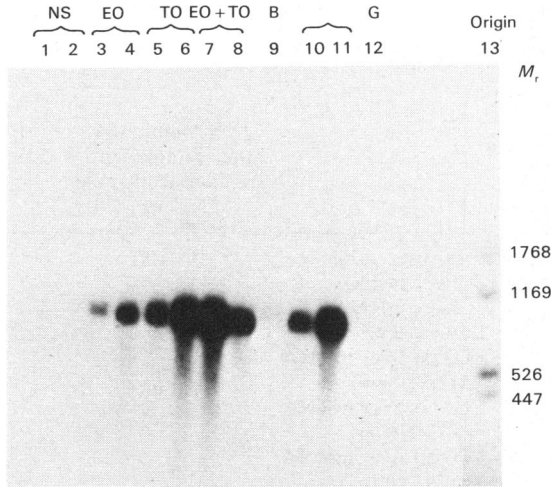

Fig. 4. *Induction of α_1-AGP mRNA in rat liver by 17α-ethynyloestradiol and turpentine oil*
The RNA blot hybridization analysis was performed after electrophoresis in 1.5% agarose gel of total poly(A)-containing RNA denatured with glyoxal. The hybridization was performed with the ^{32}P-labelled cloned DNA probe. Liver mRNA from unstimulated rat (NS): lane 1, 1.2 μg; lane 2, 4 μg. Liver mRNA from 17α-ethynyloestradiol (EO)-treated rat: lane 3, 1.2 μg; lane 4, 4 μg. Liver mRNA from turpentine-oil (TO)-injected rat: lane 5, 1.2 μg; lane 6, 4 μg. Liver mRNA from 17α-ethynyloestradiol- and turpentine-oil (EO+TO)-injected rats: lane 7, 4 μg; lane 8, 1.2 μg. Liver mRNA from normal baboon liver (B): lane 9, 1.2 μg. Two different fractions of liver mRNA from (EO+TO) rats, obtained after sucrose-density (5–20%)-gradient centrifugation: lane 10, 2.4 μg; lane 11, 0.75 μg. Globin mRNA (G): lane 12, 1.2 μg. The M_r markers used are *Hin*dIII restriction-endonuclease fragments of simian-virus-40 DNA that were labelled with [α-^{32}P]ATP at the 3′-end (lane 13): fragments of M_r 1169 and 1101 are not resolved here, and the fragment of M_r 215 is not visible on the photograph.

Table 2. *mRNA-dependency of the translation system*

Poly(A)-containing RNA was extracted from normal livers, or from livers of rats treated with 17α-ethynyloestradiol (EO), or turpentine oil (TO), or both (TO+EO), then translated in a reticulocyte-lysate cell-free preparation. Incorporation of [^{35}S]methionine into total translation product was measured after 2h incubation at 30°C in a 25 μl routine assay as described in the Materials and methods section. Each value represents the average of three samples.

Source of rat liver mRNA	Quantity of poly(A)-containing RNA ...	10^{-6} × Incorporation of [^{35}S]methionine into total translation product of rat mRNA (c.p.m.)		
		0.1 μg	0.2 μg	0.4 μg
Normal		0.83	1.5	2.9
EO		0.90	1.6	2.7
TO		0.7	1.5	2.8
TO+EO		0.84	1.6	3

Table 3. *Synthesis of α$_1$-AGP in vitro with rat liver mRNA*

Polypeptides were precipitated with 11% trichloroacetic acid in 25 μl of the translation mixture. The specific immunoprecipitates were measured as described in the Materials and methods section. Abbreviations are as in Table 2.

$$\text{Percentage of } \alpha_1\text{-AGP immunoprecipitable} = \frac{\alpha_1\text{-AGP immunoprecipitated} - \text{reference (globin mRNA)}}{\text{total trichloroacetic acid-precipitable polypeptides}}$$

	[^{35}S]Methionine precipitable with trichloroacetic acid (c.p.m.)	[^{35}S]Methionine immunoprecipitable with α$_1$-AGP (c.p.m.)	Percentage of α$_1$-AGP immuno-precipitable	α$_1$-AGP after densitometric analysis of autoradiographs	
				A	Change (fold)
Normal rat liver mRNA	5×10^5	1.1×10^3	Traces	0.025	1
(EO) rat liver mRNA	–	14×10^3	2.6	0.28	11
(TO) rat liver mRNA	–	26.5×10^3	5.1	0.71	28
(TO+EO) rat liver mRNA	–	41×10^3	8	1	40
(TO+EO) rat liver mRNA +α$_1$-AGP (1 μg)	–	20×10^3	3.8		
(TO+EO) rat liver mRNA +α$_1$-AGP (10 μg)	–	1.2×10^3	Traces		
(TO+EO) rat liver mRNA +α$_1$-AGP (100 μg)	–	10^3			
Globin mRNA	–	10^3			

are approximate, since the normal rat liver has a very low content of α$_1$-AGP mRNA.

Discussion

The serum concentration of rat α$_1$-AGP is markedly increased when a single injection of 17α-ethynyloestradiol is administered at a pharmacological dose, and repeated doses do not significantly alter the increase in serum α$_1$-AGP obtained after a single injection. Large doses of 17α-ethynyloestradiol act directly on the liver via hepatic oestrogen receptors (Lax *et al.*, 1983), and continuous exposure to 17α-ethynyloestradiol results in maximal redistribution of receptors from the cytosolic to the nuclear compartment (Kneifel & Katzenellenbogen, 1981). The dramatic increase in serum α$_1$-AGP after injection of turpentine oil in 17α-ethynyloestradiol-stimulated rats corresponds to an additive effect of the inducing agents. It suggests the presence of at least two mechanisms and/or pathways, one which is sex-steroid-inducible, and the other which is probably mediated by inflammation-induced stress factors such as interleukin-1 (Oppenheim & Gery, 1982). The increase in serum α$_1$-AGP is due to an accumulation of its mRNA in the liver cells of sex-steroid-stimulated and inflamed rats. This mRNA of 850 bases codes for a peptide of M_r 23000, and these values correspond to those indicated by Ricca *et al.* (1981) and Northemann *et al.* (1983) for the α$_1$-AGP mRNA and its translatable peptide obtained in normal and inflamed rats.

In other respects, it is noteworthy that both the glycosylation patterns of serum α$_1$-AGP from oestrogen-stimulated and inflamed rats are nearly

the same. The data suggest that the sex steroid and mediators synthesized after acute inflammation could participate in the control of the glycosylation process of the protein in an identical manner.

Findings by Baumann *et al.* (1983) indicate that glucocorticoid (dexamethasone) increases the content of α_1-AGP mRNA, a process which also occurs in adrenalectomized rats after turpentine-induced inflammation, showing that, in this case also, a second effector system could mediate an increased synthesis of α_1-AGP. Thus it appears that, as for other proteins (Houdebine *et al.*, 1978; Feigelson & Kurtz, 1978; Hager *et al.*, 1980), the hepatic synthesis of rat α_1-AGP is under complex hormonal control. However, it is not clear whether oestrogens or glucocorticoids in large doses have a major physiological role in regulating the serum concentration of α_1-AGP and that of its mRNA as compared with the role of stress-related factors.

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