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Changes in the blood level and affinity to concanavalin A of rat plasma glycoproteins during acute inflammation and hepatoma growth

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Plasma concentrations of ten individual proteins were measured by electroimmunoassay in young male Buffalo rats following injection of turpentine oil or implantation of Morris hepatoma 7777. The highest relative responses to inflammation and tumour growth were found for α_2-macroglobulin, α_1-acute-phase globulin and α_1-acid glycoprotein. As shown by crossed immuno-affinoelectrophoresis the concanavalin A-reactive fractions of the latter two glycoproteins were predominantly increased in plasma from injured and tumour-bearing rats.

It is well known that as an effect of tissue injury or tumour growth the blood concentration of certain liver-produced plasma proteins (acute-phase reactants) is greatly elevated (for references see Koj, 1974). The majority of acute-phase reactants are glycoproteins, which show affinity to plant lectins such as concanavalin A. In studies of the perfused liver isolated from injured rats we demonstrated that the radioactivity of [^{3}H]lysine incorporated into glycoproteins adsorbed on a concanavalin A–Sepharose column represents a good indicator of the acute-phase response and is well correlated with the radioactivity found in the seromucoid fraction (Koj & Dubin, 1978). α_1-Acid glycoprotein is one of the main components of the seromucoid fraction (Snyder & Ashwell, 1971). As shown by Bøg-Hansen *et al.* (1975), human α_1-acid glycoprotein exhibits heterogeneity in respect of reaction with concanavalin A during immuno-affinoelectrophoresis. Recently, Nicollet *et al.* (1981) reported that in patients with inflammatory diseases showing an increased plasma level of α_1-acid glycoprotein the concanavalin A-reactive fraction of this protein is predominantly elevated. Hence the question arises whether this particular property of α_1-acid glycoprotein is limited to human blood, and whether it is also shared by other acute-phase reactants. Here we compare the affinity to concanavalin A of rat plasma α_1-acid glycoprotein and other glycoproteins during turpentine-induced local inflammation or during growth of transplantable Morris hepatoma 7777.

Materials and methods

Reagents

Concanavalin A, concanavalin A–Sepharose 4B and Sephacryl S-300 were from Pharmacia; DEAE-cellulose 23SS (Servacel) and agarose for immunoelectrophoresis were from Serva; Tris and agarose for affinoelectrophoresis were from Fluka; Freund's complete adjuvant was from DIFCO and heparin (159 units/mg) was from Sigma. Barbitone sodium was from POCH, Gliwice, Poland. All other reagents were A.R. grade, where available.

Experimental procedure

Male Buffalo rats aged 4–5 weeks were supplied by Camura (Krakow, Poland). Animals showing any sign of disease were not used in the experiments. Local inflammation was induced by subcutaneous injection of turpentine oil (0.2 ml/100 g body weight) in the scapular region. Morris hepatoma 7777 initially obtained from the Institute of Oncology (Gliwice, Poland) was routinely transplanted by intramuscular injection into both hind legs. Material derived from approx. 1 g of tumour tissue was used for inoculation of one animal. Small samples of blood (approx. 0.1 ml) were collected at suitable intervals from the tail into heparinized test tubes and immediately centrifuged. Large blood samples required for isolation of pure proteins were obtained from the vena cava under diethyl ether anaesthesia before the animal was killed.

Purification of proteins and preparation of antisera

Pooled citrated rat plasma was used for purification of the following proteins: albumin (procedure adapted from that described for rabbit albumin by Koj & Regoeczi, 1978), fibrinogen [salting out with $(NH_4)_2SO_4$ at 25% saturation followed by molecular sieving on a column of Sephacryl S-300], α_1-acid glycoprotein (two independent methods: Charlwood *et al.*, 1976, and Shibata *et al.*, 1977),

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α_1-proteinase inhibitor and antithrombin III (a method described for horse plasma by Kurdowska *et al.*, 1982), α_1-acute-phase globulin (Urban *et al.*, 1979), α-foetoprotein (Bereta & Koj, 1982), α_1-macroglobulin and α_2-macroglobulin by the combined methods of Wong & Regoeczi (1976) and Okubo *et al.* (1981), and haptoglobin by the method of Dobryszycka & Krawczyk (1979) but with preparative polyacrylamide gel electrophoresis as the additional final step.

Monospecific antisera to these proteins were obtained by Dr. D. Stankiewicz (Medical Academy, Krakow, Poland) by immunization of rabbits according to Harboe & Ingild (1973) using Freund's complete adjuvant. In case of α_1-acid glycoprotein the protein was desialylated by heating at 80°C for 60 min with 0.01 M-H_2SO_4 in order to increase its antigenicity. Antisera to α_1-macroglobulin and α_2-macroglobulin were initially not monospecific but antibodies to the second antigen were removed by a suitable absorption. Moreover, two types of polyspecific antisera to rat proteins were obtained in rabbits using as antigens either heparinized rat plasma collected 48 h after turpentine injection, or the cell-free supernatant from tissue slices of Morris hepatoma incubated in Krebs–Ringer-bicarbonate solution as described elsewhere (Bereta & Koj, 1982).

For some immunoelectrophoretic experiments antibodies were partly purified by salting out of the antisera with $(NH_4)_2SO_4$ followed by ion-exchange chromatography on DEAE-cellulose (Harboe & Ingild, 1973). Final preparations were dialysed against citrate/saline/NaN_3 (Koj, 1980) and either kept at +4°C (for direct use) or stored frozen at −20°C for longer intervals of time.

Immunoelectrophoretic techniques

Quantitative determinations of individual rat plasma proteins were carried out by electroimmunoassay (rocket immunoelectrophoresis of Laurell, 1965) on 9 cm × 9 cm glass plates in 1% agarose containing suitable antibodies (Weeke, 1973*a*; cf. Fig. 2). As a rule estimations were repeated twice and the amount of a given protein in the analysed plasma was evaluated by comparison with serial dilutions of the purified protein.

Changes in the protein pattern elicited by local inflammation or tumour growth were evaluated by means of two-dimensional crossed immunoelectrophoresis of Minchin-Clarke & Freeman (1968) as described by Weeke (1973*b*) using either polyspecific antisera or a mixture of monospecific antisera. Affinity of plasma glycoproteins to concanavalin A was evaluated by immuno-affinoelectrophoresis as described by Bøg-Hansen *et al.* (1975). The agarose gel used in the first dimension run contained usually 0.5 mg of concanavalin A/ml ($130\,\mu g/cm^2$) and 5×10^{-4} M of each of $MgCl_2$, $MnCl_2$ and $CaCl_2$, while monospecific or polyspecific antisera were incorporated into the second dimension agarose gel.

Miscellaneous methods

Protein content was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Quantification of the area under immunoprecipitation peaks was done by drawing the peak on tracing paper, cutting out the area and weighing.

Results

Kinetics of changes in the concentration of plasma proteins during acute inflammation and hepatoma growth

By using rocket immunoelectrophoresis we analysed changes in plasma levels of 10 individual proteins while the total protein content was measured by the Lowry method. The following values [mean(mg/ml) ± S.E.M., five to seven animals] were found in the plasma of apparently healthy male Buffalo rats aged 4–5 weeks: total protein, 61.1 ± 2.35; albumin, 26.2 ± 0.59; fibrinogen, 2.75 ± 0.28; haptoglobin, 1.58 ± 0.27; α_1-acute-phase globulin, 0.94 ± 0.10; α_1-proteinase inhibitor, 1.10 ± 0.09; antithrombin III, 0.42 ± 0.05; α_1-acid glycoprotein, 0.09 ± 0.01; α_1-macroglobulin, 1.85 ± 0.25. The level of α_2-macroglubulin could be expressed only in relative terms since at the time of experiments we did not have at our disposal the purified antigen. The α-foetoprotein level in tumour-bearing rats measured on the 21st day after implantation ranged from 2.2 to 8.1 mg/ml (mean 4.45 ± 0.95). The observed level of α_1-acute phase globulin is higher than that reported by Urban *et al.* (1979) while that of α_1-macroglobulin is considerably below that given by Bosanquet *et al.* (1976). However, these discrepancies may be related to differences in the strain, age or sex of the examined rats. It should be noted that the level of other acute-phase reactants such as α_1-acid glycoprotein, haptoglobin or fibrinogen was in our control animals within the range reported by other authors (Shibata *et al.*, 1977; Franks *et al.*, 1981).

In agreement with previous findings (cf. Koj, 1974) we observed that aseptic inflammation evoked by injection of turpentine into rats led to a fast increase in plasma concentrations of several proteins. The most affected were α_2-macroglobin, α_1-acid glycoprotein, α_1-acute-phase globulin, haptoglobin and fibrinogen (Fig. 1). In contrast to these proteins albumin concentration in plasma decreased to a minimum on the second day. This phenomenon did not compensate for increased formation of acute-phase reactants and the total protein contents

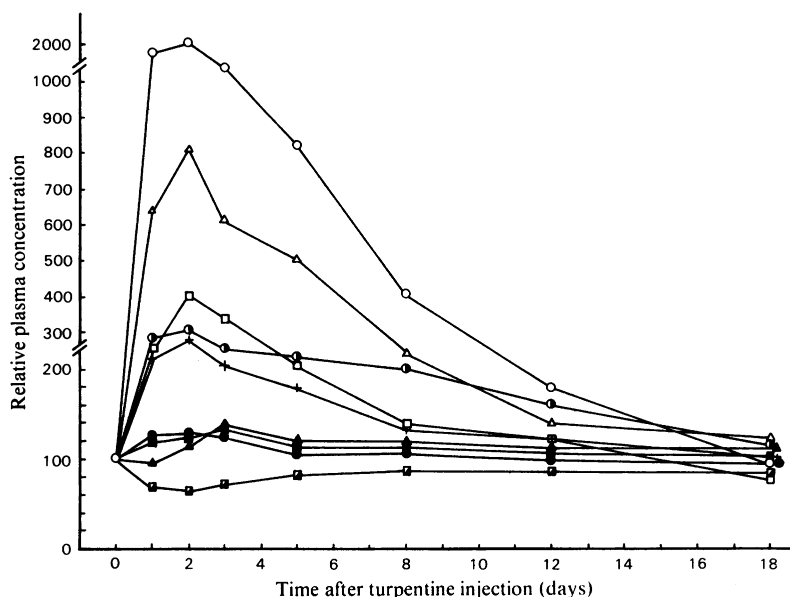

Fig. 1. *Time course of changes in rat plasma level of some proteins following subcutaneous injection of turpentine oil*
Determinations were made by means of rocket immunoelectrophoresis using monospecific antisera and purified rat proteins as standards. Relative values are reported assuming the initial plasma concentration of a given protein at time 0 as equal to 100. ○, α_2-macroglobulin; △, α_1-acid glycoprotein; □, α_1-acute phase globulin; ◑, haptoglobin; +, fibrinogen; ●, α_1-proteinase inhibitor; ■, antithrombin III; α_1-macroglobulin; ◪, albumin.

in the plasma remained augmented by 10 to 20% in comparison with the initial values. As shown in Fig. 1 the return to normal plasma levels of the majority of proteins examined was slow and extended over more than 10 days after challenge with turpentine.

Transplantation of Morris hepatoma led to a biphasic change in concentration of the majority of the plasma proteins (Figs. 2 and 3). The early rise of the acute-phase reactants (maximum at 2–4 days) probably represents the inflammatory reaction to intramuscular injection of tumour tissue. Most of the injected hepatoma tissue is destroyed by the recipient response, as witnessed by the disappearance of α-foetoprotein which was usually detectable on the second but not on the fourth day after inoculation (Fig. 2*d*). The second phase of the rise of acute-phase reactants is related to the growth of the tumour and is accompanied by reappearance and fast rise in the recipient blood of hepatoma-produced α-foetoprotein. Visually detectable tumours appeared on the hind legs between 6 and 10 days after implantation and afterwards they steadily increased in size until the death of the animal, usually after 22–30 days. At that time the weight of the tumour often exceeded 30 g, corresponding to 25% of the total body weight of the animal. The total protein concentration in the plasma of tumour-bearing rats remained almost constant during the whole period of observation. We decided to exclude from our report the terminal stages of tumour growth after day 21 of implantation because they were often complicated by additional symptoms of disease (diarrhoea, infections and abscesses developing in necrotic tumour areas).

Alterations in the immunoelectrophoretic protein pattern of rat plasma during acute inflammation and hepatoma growth

Significant changes in plasma concentrations of the individual proteins depicted in Figs. 1–3 were also observed during crossed immunoelectrophoresis, confirming the observations of Abd-el-Fattah *et al.* (1981). With the use of a polyspecific antiserum to rat plasma the picture was rather complicated, since over 15 separate protein peaks could be distinguished. A clearer pattern was obtained with the antiserum to rat hepatoma proteins while the best resolution of several antigens migrating in the albumin region could be achieved by incorporation of concanavalin A into the first-dimension agarose gel (Figs. 4*b*–4*d*). During affinoelectrophoresis some glycoproteins, including macroglobulins, are tightly bound to the lectin forming a rocket-like precipitate, while others are delayed to a variable extent and, after migrating into the antibody-containing gel, exhibit marked heterogeneity. The most conspicuous multiple peaks in Fig. 4 belong to α_1-acute phase globulin, α_1-proteinase

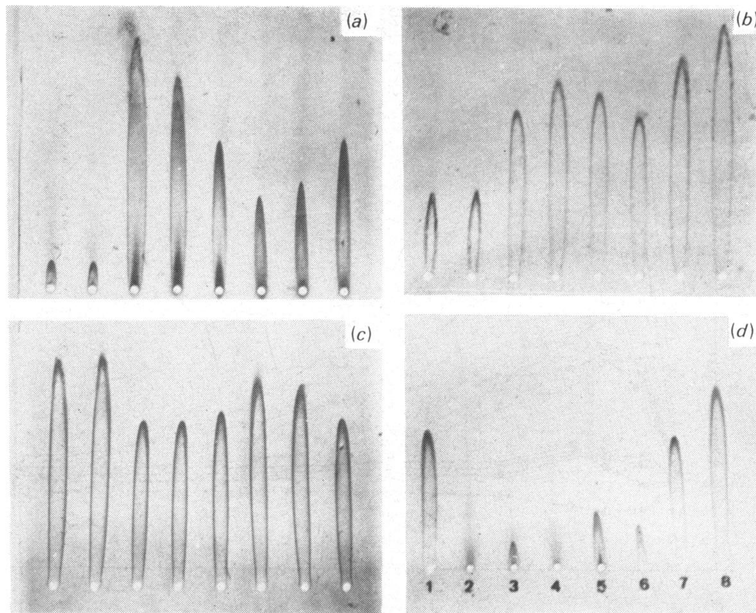

Fig. 2. *Typical plates showing changes in plasma level of four proteins in hepatoma-bearing rats as determined by rocket immunoelectrophoresis*

(*a*) α_2-macroglobulin; (*b*), α_1-acute phase globulin; (*c*) albumin; (*d*), α-foetoprotein. On plates (*a*)–(*c*) wells 1 and 2 contained duplicate plasma samples taken before tumour implantation, and wells 3–8 contained samples taken on days 2, 4, 7, 12, 18 and 21, respectively. On plate (*d*), well 1 contained α-foetoprotein standard (0.5 μg of protein), well 2 contained plasma taken before tumour implantation, wells 3–5 contained plasma taken after 2, 4 and 7 days (all undiluted), and wells 6–8 contained 10-fold diluted plasma taken 12, 18 and 21 days after tumour implantation.

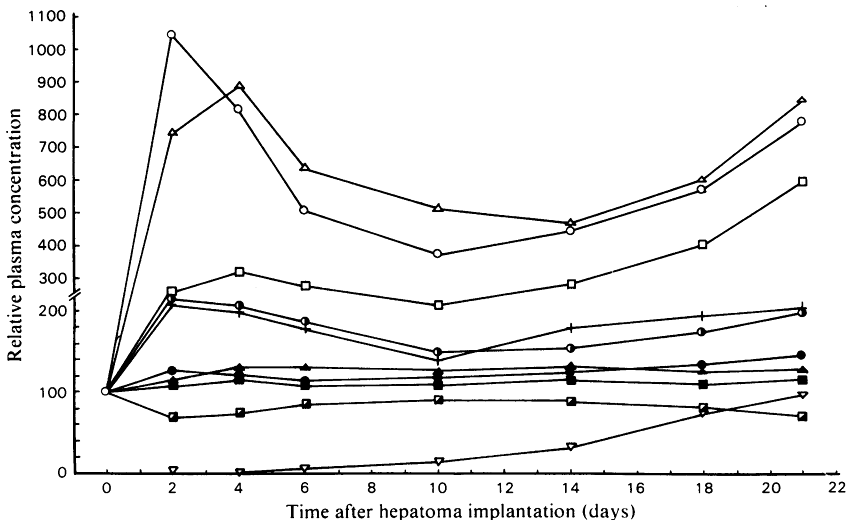

Fig. 3. *Time course of changes in rat plasma level of some proteins following implantation of Morris hepatoma 7777*

Explanations are as in Fig. 1 except that the highest α-foetoprotein (▽) level (4·45 mg/ml) was assumed as 100.

inhibitor and α-foetoprotein, each showing three (or more) components differing in affinity to concanavalin A. The separation of these components is not complete except for the fastest one corresponding to the concanavalin A-non-reactive form of α_1-acute phase globulin. After arbitrary division of

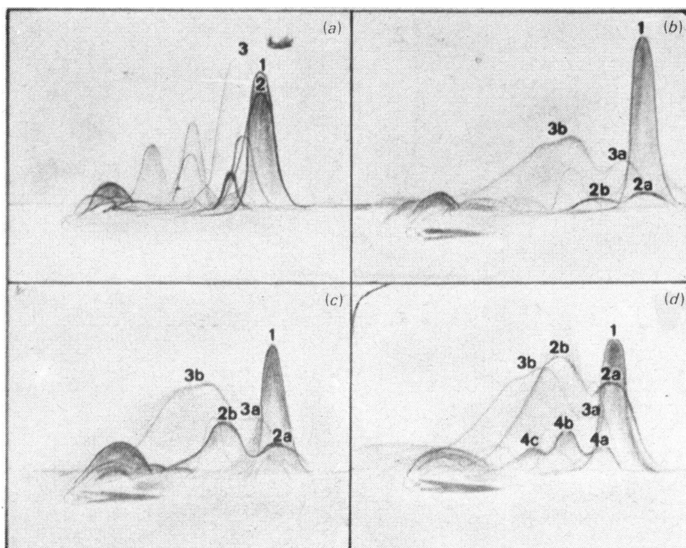

Fig. 4. *Heterogeneity of some rat plasma proteins observed during crossed immuno-affinoelectrophoresis (plates b–d)*
For comparison the result obtained during crossed immunoelectrophoresis (*a*) is also shown. The wells contained 2 μl of plasma: (*b*), from control rat; (*a*) and (*c*) from rat injected 48 h earlier with turpentine, and (*d*), from rat carrying Morris hepatoma 21 days after implantation. Agarose gel used in the first dimension (run from left to right) contained on plates (*b*)–(*d*) concanavalin A (0.5 mg/ml), while that used in the second direction (run from bottom to top) contained in all cases a polyspecific antiserum to the cell-free supernatant from Morris hepatoma incubate (0.2 ml per plate). 1, Albumin; 2, α_1-acute phase globulin; 3, α_1-proteinase inhibitor.

Table 1. *Relative abundance of the concanavalin A-reactive fractions in three glycoproteins of rat plasma as estimated after crossed immuno-affinoelectrophoresis*
Plasma levels of the glycoproteins were measured independently by rocket immunoelectrophoresis and are given as mg/ml ($\pm$S.E.M., $n = 6$). The relative values of abundance are in each case the mean of six electrophoretic runs (0.5 mg of concanavalin/ml of agarose gel) and were calculated as percentages of the whole peak area (cf. Figs. 4 and 5). In parentheses the scatter of results is reported.

	Source of plasma			
Protein	Rat	Time (days)	Plasma level (mg/ml)	Concanavalin A-reactive fraction (%)
α_1-Acid glycoprotein	Control	—	0.09 ($\pm$0.01)	57.2 (53–61)
	Turpentine-injected	2	0.74 ($\pm$0.08)	78.1 (67–82)
	Hepatoma-bearing	21	0.76 ($\pm$0.12)	75.6 (64–80)
α_1-Acute-phase globulin	Control	—	0.94 ($\pm$0.11)	58.6 (53–63)
	Turpentine-injected	2	3.85 ($\pm$0.28)	69.7 (64–78)
	Hepatoma-bearing	21	5.73 ($\pm$0.36)	67.6 (58–74)
α_1-Proteinase inhibitor	Control	—	1.10 ($\pm$0.09)	83.5 (77–88)
	Turpentine-injected	2	1.43 ($\pm$0.11)	85.7 (80–89)
	Hepatoma-bearing	21	1.48 ($\pm$0.18)	83.8 (79–86)

the area under the protein peak it was possible to estimate the relative proportion of these fractions, as summarized in Table 1.

The immuno-affinoelectrophoretic pattern of some other glycoproteins in rat plasma was best analysed with a suitable mixture of monospecific antisera using albumin as the reference protein (Figs. 5 and 6). α_1-Acid glycoprotein was barely detectable in control plasma, but it considerably increased in turpentine-injected or tumour-bearing rats; this concerned especially the concanavalin A-reactive components (cf. also Table 1). Although haptoglobin showed some heterogeneity during immuno-affinoelectrophoresis the separation of the components was poor and it was impossible to evaluate changes in relative proportions of these components

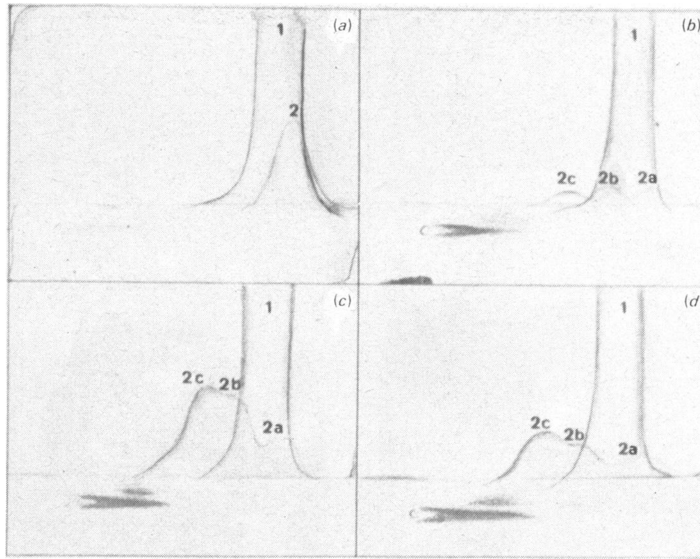

Fig. 5. *Heterogeneity of rat plasma α_1-acid glycoprotein observed during crossed immuno-affinoelectrophoresis (plates b–d)*
For comparison the picture obtained during immunoelectrophoresis is also shown (*a*). The well contents and agarose composition used in the first dimension are as in Fig. 4. The agarose gel used in the second dimension contained in all cases a mixture of the monospecific antiserum to rat albumin (0.1 ml) and monospecific antiserum to rat α_1-acid glycoprotein (0.2 ml). 1, Albumin; 2, α_1-acid glycoprotein.

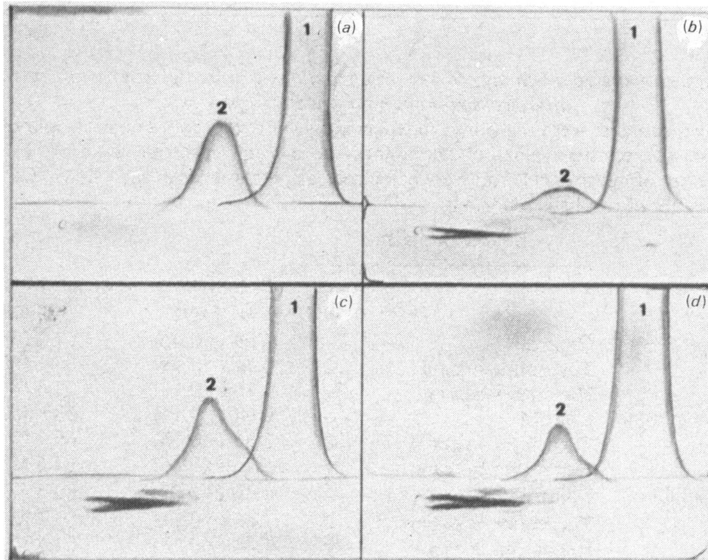

Fig. 6. *Heterogeneity of rat plasma haptoglobin observed during crossed immuno-affinoelectrophoresis (plates b–d)*
For explantions see the legends to Figs. 4 and 5. The agarose gel used in the second dimension run contained in all cases a mixture of monospecific antiserum to rat albumin (0.1 ml) and monospecific antiserum to rat haptoglobin (0.2 ml). 1, albumin; 2, haptoglobin.

during the acute-phase response or during tumour growth (Fig. 6). Similar conclusions were reached with fibrinogen or antithrombin III (results not shown). The heterogeneity of α-foetoprotein observed during immuno-affinoelectrophoresis of plasma from tumour-bearing rats (Fig. 4*d*) was similar to that

observed during analysis of the Morris hepatoma incubate (Bereta & Koj, 1982).

Discussion

Although the rat is often used in studies of changes of the plasma protein pattern in various pathological conditions the interpretation of results is difficult because of a rather complex electrophoretic picture of the α_1-globulin region due to the presence of some proteins not detected in human serum, such as α_1-macroglobulin and α_1-acute phase globulin. As demonstrated by Bosanquet *et al.* (1976) the former is only slightly increased in injured rats, in distinction to α_2-macroglobulin. On the other hand, α_1-acute phase globulin, first described by Darcy (1964) and later characterized by Gordon & Louis (1969) and by Urban *et al.* (1979) is a major acute-phase reactant in rat plasma. This protein is sometimes confused with another typical acute-phase reactant, α_1-acid glycoprotein, though their immunological and physicochemical properties or amino acid composition bear no resemblance. Having at our disposal monospecific antisera to α_1-macroglobulin, α_2-macroglobulin, α_1-acute phase globulin and to α_1-acid glycoprotein we could simultaneously follow their changes in the inflammatory and neoplastic sera.

The results obtained (Figs. 1–3) support the observations of other authors (cf. Abd-el-Fattah *et al.*, 1981) that changes in the plasma protein pattern during tumour growth resemble to some extent those following tissue injury, although they develop at a slower rate. The biphasic curves given by the plasmas from the tumour-bearing and injured rats, Fig. 3, are most different in respect to α_1-acute phase globulin and α_2-macroglobulin. The former reaches the highest level in the terminal stages of tumour growth, while the latter responds more effectively to turpentine-induced inflammation or to injury elicited by inoculation of the tumour. These differences may have several possible explanations, e.g. the hormonal status of the animal. It is known that the synthesis of α_2-macroglobulin critically depends on adrenal steroids (Heim & Ellenson, 1967; Weimer & Goggshall, 1967) whereas α_1-acute phase globulin is synthesized effectively by the liver of adrenalectomized rats (Gordon & Koj, 1968). Insufficiency of the adrenal cortex is likely to occur in the terminal stages of a malignant disease. In addition, one has to take into account a possible contribution of the tumour to the rat plasma protein pool, since Morris hepatoma 7777 during incubation *in vitro* synthesizes at least some of these proteins (Koj, 1980; Bereta & Koj, 1982). However, as reported by Schreiber *et al.* (1966), Morris hepatoma is unable to secrete into the bloodstream *in vivo* measurable quantities of plasma albumin.

When comparing the response of individual rat plasma proteins to tissue injury it is clear that the term 'acute-phase reactants' is not sufficiently precise. The best indicators of acute inflammation and tumour growth in the rat are α_2-macroglobulin, α_1-acid glycoprotein and α_1-acute phase globulin which show a 5–20-fold increase in comparison with the plasma level of healthy animals. The relative responses of fibrinogen and haptoglobin are less spectacular; their concentrations go up approx. 3-fold. The other glycoproteins of liver origin examined (α_1-macroglobulin, α_1-proteinase inhibitors and antithrombin III) increase only moderately (less than 50%) and they should not be regarded as true acute-phase reactants in the rat. However, in terms of absolute concentration in plasma α_1-acute phase globulin, fibrinogen and haptoglobin show the most conspicuous changes, their maximum level being higher by 3–6 mg/ml than the normal concentration, while the rise of α_2-macroglobulin or α_1-acid glycoprotein does not usually exceed 1 mg/ml. Among all proteins examined albumin is most affected, its level being reduced by 8–9 mg/ml.

The biphasic response of albumin and acute-phase reactants to tumour implantation was observed by several authors, including Weimer *et al.* (1967) and Abd-el-Fattah *et al.* (1981). The magnitude of the early inflammatory reaction depends on many factors, such as the amount of malignant tissue injected, implantation procedure, immunological reaction and eventual accompanying infection. In our experiments this early response was significant but the rats were injected with relatively large amounts of tumour tissue to avoid implantation failure and to ensure prompt growth of the tumour. It should be stressed that at later stages of hepatoma growth changes in the plasma level of fibrinogen and haptoglobin were often erratic, as reflected by high S.E.M. values. This may be related to intravascular clotting or haemolytic incidents known to accompany tumour invasion; such phenomena would lead to temporary reduction of plasma fibrinogen and haptoglobin. Anomalous behaviour of the haptoglobin level in plasma was also noted by Weimer *et al.* (1967) during growth of Walker carcinoma in rats. For this reason fibrinogen and haptoglobin appear to be not very reliable indicators of the progress of hepatoma growth in Buffalo rats.

Crossed immuno-affinoelectrophoresis is a powerful tool for detecting microheterogeneity in glycoproteins, human α_1-acid glycoprotein and α-foetoprotein being the most extensively studied proteins so far. Recent investigations relate structural variants of human α_1-acid glycoprotein to certain physiological and pathological states: the concanavalin A-non-reactive form prevails in plasma in pregnancy and some liver diseases (Wells *et al.*, 1981) while inflammatory states cause the rise of the

concanavalin-reactive fraction (Nicollet *et al.*, 1981). Our experiments extend these observations to rat plasma and confirm a considerable increase of the concanavalin A-reactive fractions of α_1-acid glycoprotein during the acute-phase response and during hepatoma growth (Table 1). It should be stressed here that the microheterogeneity of glycoproteins shown in Figs. 4–6 is not related to the addition of heparin to the blood, since almost identical results were obtained with citrated plasma or serum.

The data of Table 1 indicate that, as with α_1-acid glycoprotein, also α_1-acute phase globulin responded to injury by a shift toward the concanavalin A-reactive forms. On the other hand, α_1-proteinase inhibitor showed rather negligible changes in the proportion of components analysed after turpentine injection or during tumour growth. One of the differences between α_1-proteinase inhibitor and the two other glycoproteins is that its plasma level changes only little following injury. A 5–10-fold increase in the blood concentration of α_1-acid glycoprotein and α_1-acute phase globulin results from a proportional rise in their rates of synthesis in the liver, as already demonstrated by various authors (Gordon & Koj, 1968; Jamieson *et al.*, 1975). Recent studies indicate that the intrahepatic precursor of rat α_1-acid glycoprotein is more rich in mannose and neutral sugars than its mature serum form and it shows a high affinity for concanavalin A (Friesen & Jamieson, 1980; Nagashima *et al.*, 1980). It is tempting to speculate that during the greatly increased synthesis caused by acute inflammation or tumour growth processing of the α_1-acid glycoprotein precursor in the liver cell is impaired and 'unfinished' molecules are secreted to the blood, representing the concanavalin A-reactive fraction

However, one should consider an alternative explanation. α_1-Acid glycoprotein, at least in man, exists in several genetically determined variants (Berger *et al.*, 1980) differing in amino acid substitutions and in composition of carbohydrate chains (Fournet *et al.*, 1978; Schmid *et al.*, 1979). If such microheterogeneity also occurs in rat, the acute-phase response may lead to preferential stimulation of the synthesis of the concanavalin A-reactive form. A similar explanation may apply also to α_1-acute-phase globulin; its heterogeneity on isoelectric focusing has already been demonstrated by Gordon & Louis (1969). On the other hand, the results of our experiments do not provide any evidence for tumour-dependent modification of the glycoproteins examined although they are synthesized by Morris hepatoma *in vitro* (A. Koj, A. Dubin & H. Kasperczyk, unpublished work).

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