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Journal of Chromatography B, 771 (2002) 107–130

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Review

Monitoring the effects of drug treatment in rat models of disease by serum protein analysis

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Abstract

In this review we list from literature investigations on rat serum proteins using electrophoretic techniques in connection with drug testing. From our own research work, we provide annotated two-dimensional maps of rat serum proteins under control and experimental conditions. Emphasis is on species-specific components and on the effects of acute and chronic inflammation. We discuss our project of structural proteomics on rat serum as a minimally invasive approach to pharmacological investigation, and we outline a typical experimental plan for drug testing according to the above guidelines. We then report in detail on the results of our trials of anti-inflammatory drugs on adjuvant arthritis, an animal model of disease resembling in many aspects human rheumatoid arthritis. We demonstrate a correlation between biochemical parameters and therapeutic findings and outline the advantages of the chosen methodological approach, which proved also sensitive in revealing ''side effects'' of the test drugs. In an appendix we describe our experimental protocol when performing two-dimensional electrophoresis of rat serum.

Keywords: Reviews; Drug treatment effects monitoring; Proteins

Contents

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1570-0232/02/$ – see front matter
PII: S0378-4347(01)00562-X

1. Introduction

Among serum components, the different classes of immunoglobulins derive from B-lymphocytes whereas most of the other major proteins are produced by hepatocytes. The secretion repertoire is much more complex for liver than for any other organ; various messengers, including hormones and cytokines, may modulate the biosynthesis of its different products. These messengers are produced by peripheral organs, according to patterns that vary with physiological (e.g. sex, age) and pathological condition. By conveying information they induce adaptive responses in which the synthesis of different proteins is differentially affected to comply with the needs of the peripheral organs. For these reasons the protein setup in serum is an indicator of the condition of the whole organism. Changes from ''reference'' are brought about in a complex way. Hence, the interpretation of the actual pattern requires: (i) a reliable set of ''test'' data on serum proteins, as typically provided at the qualitative and quantitative level by two-dimensional electrophoresis (2-DE), to be compared with (ii) a comprehensive database, i.e. an extensive collection of ''reference'' patterns under defined (experimental) conditions.

The different theoretical considerations discussed above have found practical application to a variable extent. Serum is indeed the specimen of choice for analysis in clinical chemistry. When dealing with human patients a further selection criterion applies, and indeed prevails, namely minimising discomfort caused by sampling; in this respect serum has the obvious advantage of requiring a minimally invasive procedure for its collection. Practical considerations apply also to the selection of the typical procedures for serum analysis: in clinical chemistry laboratories mainly economical reasons have so far restricted them to cellulose acetate electrophoresis and immuno-nephelometry.

Research laboratories are taking a different direction. High-resolution analytical techniques are preferred, and among them 2-DE is becoming more and more common. Conversely, sampling procedures are not a concern when in vivo experimental work is on laboratory animals. As a result, most published data on rat proteomes deal with tissue samples (see Tables 1 and 2), whereas serum analysis has received comparatively little attention.

2. Rat Serum Protein Study Group: our own research plan

Contrary to this trend, our group has devoted a detailed investigation to all the basic aspects of proteomics of rat serum and, more recently, of rat urine and cerebrospinal fluid. Our studies included

- as for *structural proteomics* aspects:
  - 2-DE according to IPG-DALT protocols (see Appendices A and B), either with wide (nonlinear 4–10 [1]) or with narrower (1–3 pH units [2,3]) gradients;
  - identification of peptides in all major spots with immunological and mass-spectrometry techniques; for this we had invaluable support from Washington University, Seattle, USA [4–6];
- as for *functional proteomics* aspects:
  - quantification of spot volumes under varying experimental conditions [6–10];
  - construction of sql databases of the quantitative evaluations, that may be interrogated on a server–client basis;
- as for *informatics/knowledge diffusion* aspects:
  - publication of our main findings in a web site, partially federated to Swiss-2DEPAGE (http://www.expasy.ch/ch2d), at http://linux.farma.unimi.it [11]. Figs. 1 and 2 correspond to the two annotated maps for rat

Table 1
On-line databases on rat proteins

Site name	Institution	Web address
Rat Serum Protein Study Group	Università degli Studi Milano, Veterinärmedizinische Universität Wien, University of Washington Seattle, Imperial College School of Medicine, London	http://linux.farma.unimi.it
RAT HEART-2DPAGE: Two-dimensional polyacrylamide gel electrophoresis database of rat heart	German Heart Institute Berlin	http://gelmatching.inf.fu-berlin.de/~pleiss/2d/
HSC-2DPAGE: 2-DE Gel Protein Databases at Harefield	Heart Science Centre, Harefield Hospital	http://www.harefield.nthames.nhs.uk/nhli/protein/
TOOTPRINT: two-dimensional gel database for dental tissues	University of Otago, Dunedin	http://bioc111.otago.ac.nz:591/tooth/home.htm

serum proteins (control and inflammation pattern); clicking on their spots opens links to the specific entries in SWISS-PROT (http://www.expasy.ch/sprot/) or OWL (http://prowl.rockefeller.edu/in) databases, which list protein names, give detailed information about sequences, and provide a summary of biological properties.

As a first and main step we have defined a reference map for serum proteins in healthy adult male rats [4,5]. Our map was constructed on specimens from strain Sprague–Dawley (CD); however, a recent investigation has demonstrated that at the quali- and quantitative level differences among strains (Sprague–Dawley, Lewis and Wistar Kyoto) are minimal, at least under baseline conditions [6]. A difference in migration is seen for haptoglobin β-chains (Hp): in Lewis (LW) the resolution from α_1-macroglobulin is higher than in Sprague–Dawley rats [7,9] (Fig. 3). Hp, however is expressed at high levels only during acute phase reaction [4,5]. Differences in age have little influence on the pattern of adult rats, whereas a sharp change is observed between serum samples of newborns and adults [5]. Several differences are detected between males and females [7]. Thiostatin (α_1-MAP) is five times higher in females than in males, hemopexin (Hpx) almost 50% higher, C-reactive protein (CRP) and apoE ca. 25% higher in females; α_1B-glycoprotein can be detected only in female serum [6]. Gc-globulin (Gc) and retinol-binding protein (RBP) are about twice as high in males, α_2-HS-glycoprotein (α_2-HS) and α_1-antitrypsin (α_1-AT) about 25 and 30% higher in males than in females, respectively. Once expressed after inflammation, the concentration of α_2-macroglobulin is about 40%, that of oroso-mucoid ca. 30% higher in male rats.

We also investigated in much detail changes in serum protein levels associated with acute phase reaction [5,7–9]. The primary aim of this step was surveying the whole synthetic repertoire of proteins secreted from rat liver. However, this knowledge made it then possible to classify other pathological serum patterns in regard to similarity/nonsimilarity to those observed in experimental inflammation.

The protein assortment in rat serum is definitely different to that in human serum. Even common proteins display different physico–chemical parameters (primary structure, hence p*I*, M_r and glycosylation pattern). Some proteins are present in much lower concentration in rat than in human serum (e.g.

Table 2
Scientific literature on rat tissue proteomics

Topic	Keywords	References
Structural proteomics	Myosin in muscle ontogenesis	[140]
	Liver	[76,77]
	Heart	[141]
	Kidney cortex and medulla	[142]
	Cholangiocyte	[143]
	Tooth enamel	[144]
	Heart	[145]
	Dermal papilla	[146]
	Brain	[147,148]
	Hepatic stellate cells	[149]
	Soleus muscle (denervation)	[150]
Effects of diet	Brain tubulin induction by ammonium salts	[151]
	Liver and ethanol	[152]
	Brain protein phosphorylation and ethanol	[153]
	Liver keratin phosphorylation and ethanol	[154]
	Linoleic acid and liver ACMSD[a]	[155]
Effects of hormones	Insulin on adipocyte plasma membrane	[156]
	Peptide hormones on corpus luteum and adrenal cortex	[157]
	H3 and H4 on adipocytes (insulin-like)	[158]
	Triiodothyronine on heart and	[159,160]
	after hepatectomy	[161]
	Insulin on muscle P20 phosphorylation	[162]
Effects of xenobiotics	Isoproterenol and heart	[159]
	Phenobarbital and liver	[163]
	Bromobenzene and kidney biliverdin reductase	[164,165]
	Morphine and myelin MARPP(14–20)[b]	[166]
	Warfarin and liver fatty acid binding protein	[167]
	Methapyrilene[c] and liver	[108,168,169]
	Perfluorocarboxylic acids and liver enoyl-CoA hydratase	[170]
	Etomoxir[d] and liver	[171]
	Cyclosporine A and kidney	[172]
	Lead and kidney	[173]
	Puromycin aminonucleoside and kidney	[174]
	Nafenopin[e] and liver	[175]
	Lovastatin or	[176]
	fluvastatin and liver	[177]
Cancer tissues/ cell lines	Glutathione S-transferase in preneoplastic liver nodules	[178]
	Hepatoma	[179–182]

[a] ACMSD = alpha-amino-beta-carboxymuconate-epsilon-semialdehyde decarboxylase.
[b] MARPP(14–20) = morphine- and cyclic AMP-regulated phosphoproteins of 14–20 kDa.
[c] methapyrilene = a mitochondrial proliferator.
[d] etomoxir = an irreversible carnitine palmitoyltransferase I inhibitor.
[e] nafenopin = a peroxisome proliferator.

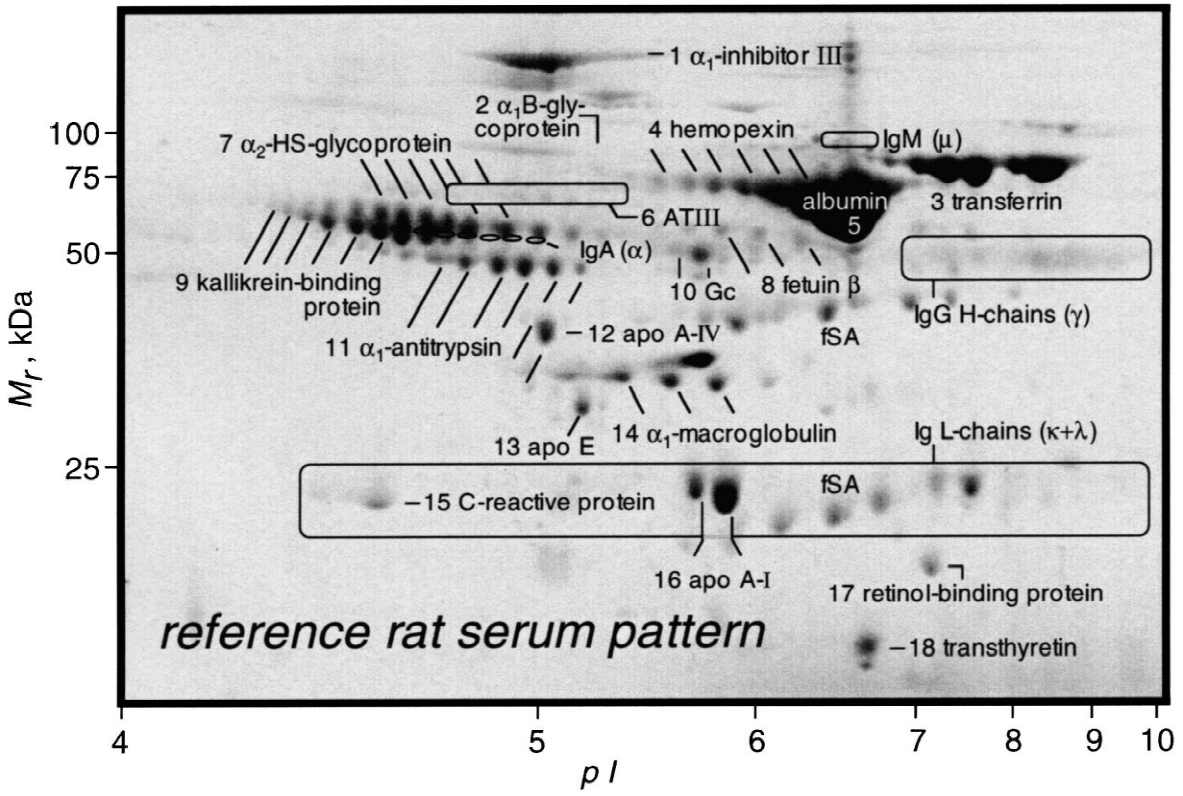

Fig. 1. 2-DE map of control rat serum (male CD). Protein identifications are marked across the pattern; entry numbers refer to the compilation in Section 3. (Map first published in Ref. [4], and regularly updated at http://linux.farma.unimi.it).

orosomucoid and haptoglobins under baseline conditions), others are present in much higher concentration (e.g. C-reactive protein); some proteins are species specific to the rat (e.g. α_1-inhibitor III, kallikrein-binding protein, α_1-macroglobulin, already expressed under baseline conditions; thiostatin and serine protease inhibitor 3, both seen only in inflammation).

3. Rat serum proteins: a survey

In the following we list all major proteins in rat serum (except immunoglobulins). The sequence is based on their position (left to right and top to bottom) in 2-DE maps, of control serum (Fig. 1) for entries 1–18, of APP serum for entries 19–24 (Fig. 2); numbering of the entries is as in Figs. 1 and 2. Most of the basic information is taken from the compilation in SwissProt; other relevant literature data and our own findings on protein behaviour in experimental inflammation are listed as well.

Two types of experimental inflammation are made reference to:

- acute inflammation brought about by i.m. injection of 0.5 ml turpentine per kg body weight; the effects were evaluated in time-course, over a 5-day period [8];
- chronic inflammation brought about by i.m. injection (into the right hind footpad) of 100 μl of a suspension of 10 mg/ml heat-killed *M. tuberculosis* in paraffin oil, which resulted in adjuvant arthritis; the effects were evaluated 14 days after immunization [9].

The effects of acute (AI) and chronic inflammation

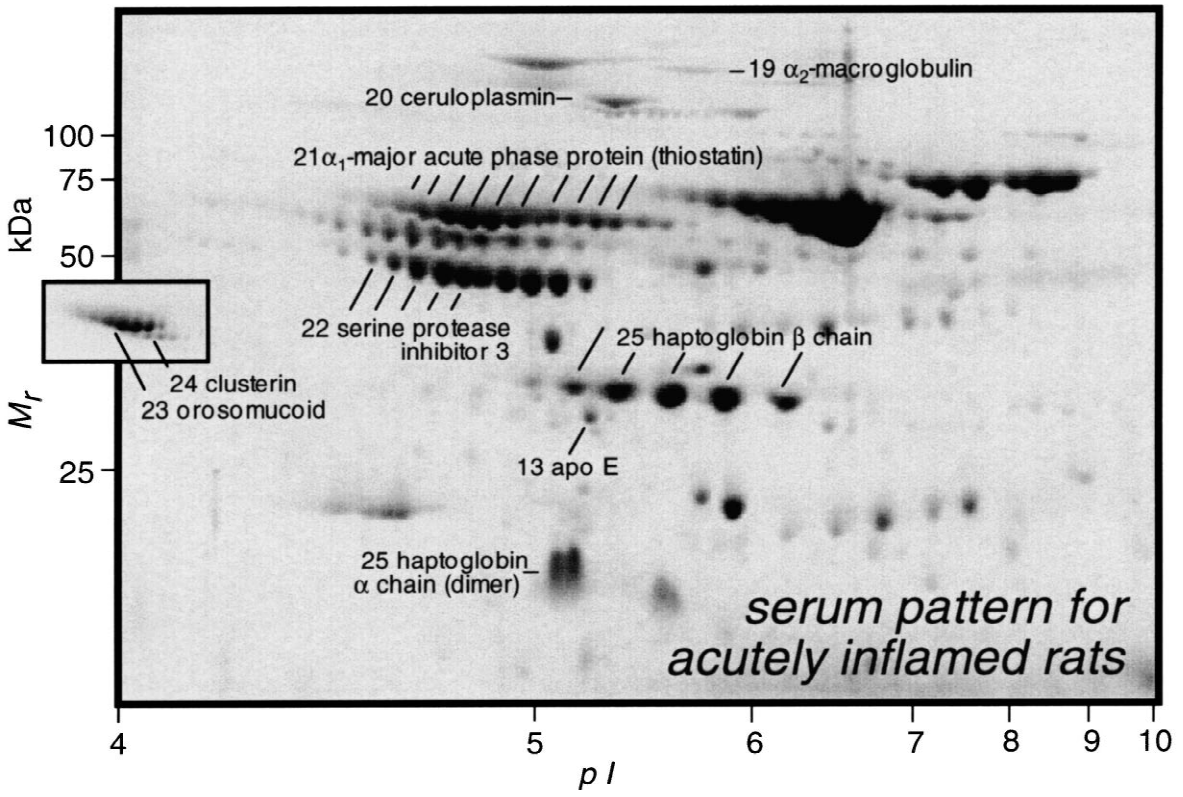

Fig. 2. 2-DE map of serum from an acutely inflamed rat (male CD, day 2 after i.m. injection of 5 ml/kg turpentine). Protein identifications are marked across the pattern; entry numbers refer to the compilation in Section 3. The inset on the left is from a 2.5–5 pH gradient. (Map first published in Ref. [5] and regularly updated at http://linux.farma.unimi.it).

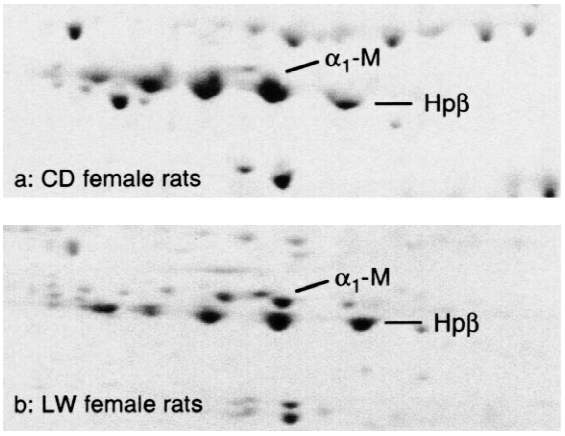

Fig. 3. Varying resolution between Hp β chains and α_1-M in serum from different rat strains. Panel a, Sprague–Dawley female rats, day 2 of acute inflammation [7]; panel b, Lewis female rats, day 14 of chronic inflammation [9].

(CI) are compared in Fig. 4[1]; data for the former refer to day 2 after treatment. AI and CI affect the same positive and negative APPs, with the notable exception of Tf, whose concentration drops by 50% in CI but appears unchanged after turpentine injection. However Tf shows no difference from baseline only on day 2, whereas its concentration decreases on days 1 and 4. The behavior of kallikrein-binding protein (KBP) is also very different (down 100% in CI vs. down 48% in AI). The time course of the expression of KBP after AI is complex,

[1]Data normalization. Differences in protein concentrations are better expressed as percent changes vs. a suitable reference. Different calculations may be devised, all of them flawed by some limitations. Contrary to the original publications, in the present report data were computed with reference to control conditions. This has the disadvantage of emphasizing the de novo synthesis of positive APPs whose baseline level is below detection limit.

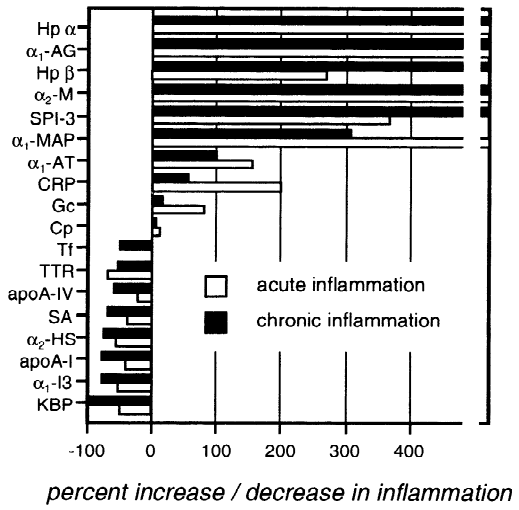

Fig. 4. Changes in concentration of some rat serum proteins after two protocols of experimental inflammation. Open bars, acute inflammation (AI), i.e. day 2 after injection of turpentine [7]; solid bars, chronic inflammation (CI), i.e. day 14 after induction of adjuvant arthritis [9]. Data from experiments on female rats. Changes are computed as: 100*(AI − C)/C for acute inflammation and 100*(CI − C)/C for chronic inflammation; C is the untreated control animal. In CD rats, Hp chain β was not resolved from from α1-M, so its increase after turpentine treatment is slightly underestimated.

with a minimum on day 1 and a maximum on day 3 [8].

Other major quantitative differences between CI and AI involve apoAI (down 77% in the former vs. only 40% in the latter) and apoAIV (down 57% vs. down 20%), albumin (down 68% vs. down 37%) and CRP (up 58% vs. up ca. 200%).

3.1. Rat serum protein database

(1) α_1-inhibitor III (α_1-I3) [12–14] is a protease inhibitor with a wide spectrum of protein targets, which attaches through its thiolester function. As an inhibitor of chymotrypsin, it is analog (but not homolog, see serin protease inhibitor 3 below) of human α_1-antichymotrypsin. Its structure is similar to other proteins of the α-macroglobulin family, including complement. The inhibitor has reduced plasma concentration in acute phase, i.e. the protein is a *negative* acute-phase reactant (APP). Under our experimental conditions, its concentration decreases by ca. 80% in CI and by ca. 50% in AI.

(2) α_1B-glycoprotein [15], a *positive* APP, is present at concentrations above detection limit only in female rats. Its structure is similar to immunoglobulin and major histocompatibility complex domain.

Afamin or α-albumin [16] comigrates with α_1B-glycoprotein. For this protein a role is hypothesized in the transport of yet unknown ligand(s). It is composed of three homologous domains, and belongs to the ALB/AFP/VDB family.

(3) Transferrin (Tf) [17–19] is an iron binding transport protein that can bind two atoms of ferric iron in association with an anion, usually bicarbonate. It is composed of two homologous domains with different affinity for iron ions. The protein is responsible for the transport of iron from sites of absorption and heme degradation to those of storage and utilization. Serum Tf may also have a further role in stimulating cell proliferation. In acute inflammation (AI), the concentration of Tf has a biphasic course: its concentration decreases on days 1 and 4, increases on day 3, while no difference from baseline is observed on day 2, in agreement with previous findings [20]. In CI, Tf concentration drops by 50%; its pattern of glycosylation changes as well (Fig. 5).

(4) Hemopexin (Hpx) [21–23] binds heme and transports it to the liver for breakdown and iron recovery, after which the free hemopexin returns to the circulation. Its structure is composed of two domains, termed after its name (hemopexin-like). Hpx is a positive APP; in AI, its level increases by ca. 50%. In 2-DE, Hpx spots are incompletely resolved from albumin even under optimal running conditions; they may be differentially revealed by immunoblotting or for their sugar content (affinity blotting with concanavalin/peroxidase).

(5) Serum albumin (SA) [24,25], the main protein of plasma/serum, has a good binding capacity for water, Ca^{2+}, Na^{+}, K^{+}, fatty acids, hormones, bilirubin and drugs. Its main function is the regulation of the colloidal osmotic pressure of blood. Its structure is composed of three homologous domains. It belongs to the ALB/AFP/VDB family. SA is a negative APP; in AI its concentration decreases by ca. 35%, in CI by ca. 70%.

SA fragments are present with a specific pattern that may be most easily recognised on the serum protein fraction soluble in 50% saturated $(NH_4)_2SO_4$

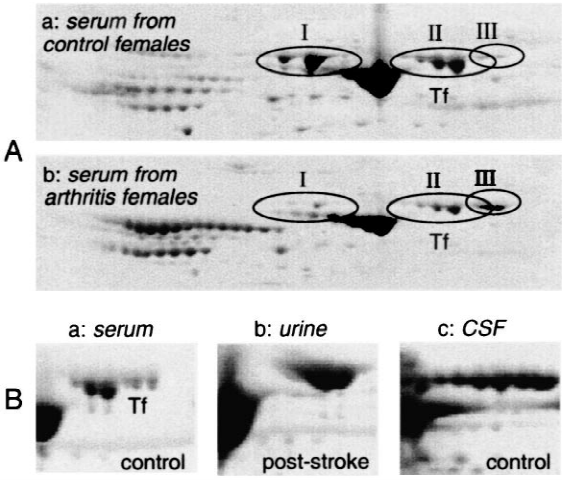

Fig. 5. Varying assortment of transferrin isoforms in different specimens. (A) Effects of chronic inflammation. In panel a (control female CD rat serum) spots marked as I correspond to iron saturated, folded Tf, spots marked as II and III to unfolded apoTf. In panel b (serum from a LW female rat with adjuvant arthritis) (i) the overall amount of Tf is decreased, (ii) the ratio I/II+III is decreased, (iii) the ratio II/III is also decreased. Close-ups of 2-DE maps [9]. (B) Differences among various biological fluids: serum (panel a), urine (panel b), cerebrospinal fluid (panel c). Samples are from healthy rats for serum and CSF, from SHRSP presenting with cerebral damage for urine [10]. The higher complexity of Tf in CSF vs. serum had already been described in humans [138,139]; no data had been previously reported for urine. In healthy humans as well as in rats, we observe a higher ratio in urine than in serum between isoforms III and II (unpublished results). Close-ups of 2-DE maps.

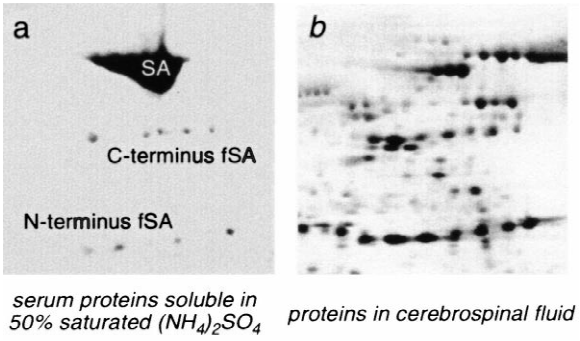

Fig. 6. Albumin fragments. C- and N-terminus proteolytic fragments, which are a dominant feature in rat CSF (close-up in panel b, control CD male) [6], may be purified from serum, together with intact albumin, after precipitation with 50% saturated $(NH_4)_2SO_4$ (close-up in panel a) (unpublished, experiments referred to in Ref. [5]).

(Fig. 6). The fragments with higher M_r have been found to correspond to the C-terminus of the molecule (peptide coverage by MS=243–602), those with lower M_r to the N-terminus (25–242) [6]. The same SA fragments are dominant features in CSF protein pattern [6].

(6) Antithrombin III (AT-III) has not been sequenced in rat genome. In humans, AT-III is the most important serine protease inhibitor (serpin) in plasma that regulates the blood coagulation cascade. AT-III inhibits thrombin as well as factors IXa, Xa and XIa. Its inhibitory activity is greatly enhanced in the presence of heparin.

(7) α_2HS-glycoprotein (α_2-HS) [26] inhibits insulin-receptor tyrosine kinase activity and insulin-stimulated receptor auto-phosphorylation while antagonising the mitogenic effect of the hormone in cultured rat hepatoma cells. α_2-HS is among the negative APPs; in AI its concentration drops by 55%, in CI by 75%. The protein belongs to the fetuin family and contains two cystatin-like domains. It undergoes complex post-translational modification involving N-glycosylation, and addition of fucose and sialic acid residues. Phosphorylation occurs at a serine residue.

(8) Fetuin-β [27] belongs to the fetuin family and contains two cystatin-like domains. The cystatins are low-molecular-mass cysteine protease inhibitors that typically comprise a domain of approximately 115 amino acids with four conserved cysteine residues known to form two disulphide bonds. Those and further conserved residues are required for the protease inhibitory capacity of these proteins. The cystatin superfamily comprises the cystatins themselves, the intracellular stefins, the kininogens and the fetuin family, which has lost cysteine protease inhibitory activity. In rats the plasma fetuin levels are low in both fetuses and adults, whereas they are highest shortly after birth in a transient fashion. Fetuins are negative APPs.

(9) Kallikrein-binding protein (KBP) [28–31] binds to and inhibits kallikreins, inhibits trypsin but not chymotrypsin or elastase. Several synonyms stress, among others, different aspects in the regulation of the expression of this protein: serine protease inhibitor 2 (SPI2), contrapsin-like protease inhibitor, thyroid hormone-regulated protein, growth hormone-

regulated proteinase inhibitor. Induction is also by sex hormones: in male rats, its level is several fold higher than in female rats. Its expression is virtually abolished by CI, whereas it changes with a complex course in AI (decrease on day 1, slight increase on day 3).

(10) Group-specific component, or vitamin D-binding protein, or Gc-globulin (Gc) [32–34] is a multifunctional protein found in plasma, ascitic fluid, cerebrospinal fluid, and urine and on the surface of many cell types. In plasma, it carries the vitamin D sterols and prevents polymerization of actin by binding its monomers. It associates with membrane-bound immunoglobulin on the surface of B-lymphocytes and with IgG Fc receptor on the membranes of T-lymphocytes. It is composed of three homologous domains and belongs to the ALB/AFP/VDB family. Its concentration is slightly affected by CI but increased by 80% by AI.

(11) α_1-antitrypsin (α_1-AT) [35,36] is an inhibitor of serine proteases and belongs to the serpin family. Its primary target is elastase, but it also has a moderate affinity for plasmin and thrombin. It is a positive APP, being increased by 100% by CI and by 157% by AI.

(12) Apolipoprotein A-IV (apoA-IV) [37–39] may have a role in chylomicrons and very low-density lipoprotein (VLDL) secretion and catabolism. It is required for efficient activation of lipoprotein lipase by apoC-II; it is a potent activator of lecithin:cholesterol acyltransferase (LCAT). ApoA-IV is a major component of high-density lipoprotein (HDL) and chylomicrons.

Nine of the 13 22-amino acid tandem repeats (each 22-mer is actually a tandem array of two, A and B, related 11-mers) occurring in its sequence are predicted to be highly α-helical, and many of these helices are amphipathic. They may therefore serve as lipid-binding domains with lecithin:cholesterol acyltransferase (LCAT) activating abilities. apoA-IV belongs to the apoA-I/apoA-IV/apoE family. Although mostly bound to lipoproteins, the protein is present in plasma also in free form, and may be lost into urine. ApoA-IV is down regulated by inflammation (−57% in CI, −20% in AI).

(13) Apolipoprotein E (apoE) [40–42] mediates binding, internalization, and catabolism of lipoprotein particles. It can serve as a ligand for the LDL (apo B/E) receptor and for the specific apoE receptor (chylomicron remnant) of hepatic tissues. The mature protein has no cysteine residues; however, in different allelic variants where cysteine residues replace arginine at positions 155 or 168, binding of apo-E to cell membrane receptors is decreased. The amino end of this protein is therefore thought to interact with the receptor. It belongs to the apoA-I/apoA-IV/apoE family.

(14) α_1-macroglobulin (α_1-M) [13,43–46] is a tetrameric assembly (ca. 7.5×10^5 Da, pI=4.4) of heterodimers; the resolved component, ca. 42 kDa, is subunit β, whereas the size of subunit α is >140 kDa. The latter has a complex structure with non-repetitive domains. α_1-macroglobulin inhibits a broad spectrum of proteinases by forming macromolecular cages inside which proteinases are cross-linked and trapped. Upon formation of a complex with proteinase, α_1-M undergoes a large conformational change that results in the exposure of its receptor-binding domain. Engagement of this domain by α-macroglobulin receptor permits clearance of the α-macroglobulin:proteinase complex from circulation. α_1-M is also an inhibitor of rat Galβ1–4GlcNAc α2-6 sialyltransferase. Its concentration is only marginally affected by inflammation.

(15) C-reactive protein (CRP) [47,48] displays several functions associated with host defense: it promotes agglutination, bacterial capsular swelling, phagocytosis, and complement fixation through its calcium-dependent binding to phosphorylcholine. In contrast to human CRP, it binds three molecules of phosphorylcholine instead of five. It belongs to the pentaxin family, and has a homopentameric structure. Pentaxins (or pentraxins) have a discoid arrangement of five noncovalently bound subunits. Two of the five chains form a dimer linked by two interchain disulfide bonds located in the C-terminal heptapeptide (specific to rat CRP). In the other three subunits, the last two cysteines are involved in an intrachain bond. CRP is a positive APP; following inflammation its concentration rises much less than in humans, i.e. by ca. 60% in CI and by ca. 200% in AI.

(16) Apolipoprotein A-I (apoA-I) [39,49] is the major protein of plasma HDL, and is also found in chylomicrons. It participates in the reverse transport of cholesterol from tissues to the liver for excretion

by promoting cholesterol efflux from tissues and by acting as a cofactor for LCAT. It belongs to the apoA-I/apoA-IV/apoE family. apoA-I is a negative APP, as its concentration is lowered by 77% in CI and by 40% in AI.

(17) Retinol binding protein (RBP) [50,51] delivers retinol from the liver stores to the peripheral tissues. In plasma, the RBP–retinol complex interacts with transthyretin (below); this prevents its loss by filtration through the kidney glomeruli.

(18) Transthyretin (TTR) [52–54] is a thyroid hormone-binding protein, most abundant in the choroid plexus but also present in the liver; it probably transports thyroxine from the bloodstream to the brain. In plasma TTR binds RBP. TTR is a homotetramer. It behaves as a negative APP, decreasing by ca. 50% in CI and by ca. 65% in AI.

(19) α_2-macroglobulin (α_2-M) [13,43] is able to inhibit all four classes of proteinases by a unique ''trapping'' mechanism. This protein has a peptide stretch, called the ''bait region'', which contains specific cleavage sites for different proteinases. When a proteinase cleaves the bait region, a conformational change is induced in the protein, which traps the proteinase. The entrapped enzyme remains active against low molecular mass (LMW) substrates whereas activity against high molecular mass (HMW) substrates is greatly reduced. Following cleavage in the bait region a thiolester bond is hydrolyzed and mediates the covalent binding of the protein to the proteinase. α_2-M is a homotetramer, which consists of two pairs of disulfide-linked chains; its structure is similar to other proteins of the α-macroglobulin family, including complement components C3, C4, and C5. By inflammatory stimulus the level of this protein first increases, then decreases after a maximum.

(20) Ceruloplasmin (Cp) [55,56] is a blue, copper-binding (six to seven atoms per molecule) glycoprotein. Four possible functions are ferroxidase activity, amine oxidase activity, copper transport and homeostasis, and superoxide dismutase activity. In its enzymatic activity, the redox reaction at the catalytic center is as follows: $4Fe^{2+} + 4H^{+} + O_2 = 4Fe^{3+} + 2H_2O$. Six Cu-ions bound per molecule act as cofactors. This protein belongs to the multicopper oxidases which contain three distinct Cu centers known as type 1 or blue, type 2 or normal, and type 3 or coupled binuclear. Cp may also play a role in fetal lung development or pulmonary antioxidant defense. It is synthesized in liver, but also in choroid plexus, yolk sac, placenta, and testis, not in stomach and small intestine. Its synthesis is induced by inflammation. Its structure contains three F5/8 type A domains, each is composed of two plastocyanin-like repeats.

(21) Thiostatin or T-kininogen I/II (precursor) or α_1-major acute-phase protein (α_1-MAP) [57–60] is the major positive APP in rat serum. Rats express four types of kininogens: the classical HMW and LMW kininogens produced by alternative splicing of the same gene, and two additional LMW-like kininogens: T-I and T-II. Kininogens are plasma glycoproteins with a number of functions: (1) as precursor of the active peptide bradykinin they effect smooth muscle contraction, induction of hypotension and increase of vascular permeability; (2) they play a role in blood coagulation by helping to position optimally prekallikrein and factor XI next to factor XII; and (3) they are inhibitors of thiol proteases. In response to an inflammatory stimulus, T-kininogen II synthesis is induced and the plasma concentration of T-kininogen I is raised. As T-kinin is preceded by a Met instead of an Arg or Lys, it is not released from its precursor by either tissue or plasma kallikrein. The structure of α_1-MAP contains three cystatin-like domains. Two genes exist for this protein, resulting from a duplication event: MAP1 and MAP2. Two types of proteins are indeed found in plasma, differing in their p*I* by about 0.3 pH units. Their baseline levels are different: α_1-MAP(2) is more abundant than α_1-MAP(1) in female rats, and is also the only form present (in very small amounts) in control males. α_1-MAP increases three times in concentration upon CI, 5.5 times upon AI.

(22) Serine protease inhibitor 3 (SPI3) [29] belongs to the serpin family. Its structure is very similar (75% identity) to that of human α_1-antichymotrypsin (homolog) but, due to a mutation in the active centre, its specificity is shifted from chymotrypsin to trypsin. It is thus an analog of α_1-antitrypsin. Its synthesis is induced by inflammation, its concentration increasing over 15 times in CI, and over 3.5 times in AI.

(23) Orosomucoid or α_1-acidic glycoprotein (α_1-AG) [61,62] appears to function in modulating the

activity of the immune system during the acute-phase reaction. α_1-AG is synthesized in the liver, the synthesis being controlled by glucocorticoids, interleukin-1 and interleukin-6; its level increases 5- to 50-fold upon inflammation. α_1-AG belongs to the lipocalin family.

(24) Clusterin or sulfated glycoprotein 2 (Sgp-2), testosterone repressed prostate message-2 (Trpm-2), dimeric acid glycoprotein (Dag), apolipoprotein J [63,64]. Its function is not yet clear; it is known to be expressed in a variety of tissues and it seems to be able to bind to cells, membranes, and hydrophobic proteins. It has been associated with programmed cell death, as it is expressed by cells undergoing apoptosis as a result of hormonal stimuli or traumatic insult. The quaternary structure is that of a disulfide-linked heterodimer. The protein is extensively glycosylated with sulfated N-linked carbohydrates.

(25) Haptoglobin (Hp) [65–67] combines with free plasma hemoglobin, preventing loss of iron through the kidneys and protecting the kidneys from damage by hemoglobin, while making the hemoglobin accessible to degradative enzymes. It is a tetramer of two α and two β chains. Although the β chain is clearly related to serine proteases, Hp has no enzymatic activity. Positions corresponding to the proteolytic active site residues of the proteases are occupied by different amino acids in haptoglobin. Hp is a positive APP, with a sharper rise in CI than in AI.

4. Rat serum proteins: biological and toxicological studies

Before our comprehensive mapping, some protein identification had been carried out after electrophoretic fractionation, either on serum itself or on the secreted products from liver slices/cultured hepatoma cells.

In these experiments serum was being fractionated with 2-DE protocols that excluded the use of dissociating/denaturing agents either from the 1 day run or from both electrophoretic steps. This option was meant to preserve, partially or completely, the quaternary assemblies typical of the structure of several serum components (above). These alternative procedures included crossed-immunoelectrophoresis [68,69], agarose electrophoresis followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under non reducing conditions [13] and IEF-PAGE fractionation [70,71]. Most serum components could be identified in the resulting patterns.

On the contrary investigations on liver and liver-secreted proteins were making use of typical 2-DE procedures (ISO-DALT [72–74]). Eight proteins, whose expression is altered by experimental inflammation, were identified among poly(A^+)-mRNA in vitro translation products from primary cultures of rat hepatocytes; eleven proteins were resolved among their secretion products (orosomucoid, α_2-macroglobulin, hemopexin, α_1-antichymotrypsin, α_1-antitrypsin, γ-fibrinogen, β-haptoglobin, amyloid-associated P component, transferrin, albumin, α_{2u}-globulin) [75]. Albumin, pro-albumin and pro-apo A-I lipoprotein could be detected in standard 2-DE maps of rat liver [76,77].

Without resorting to electrophoretic techniques, production of four proteins (albumin, α-fetoprotein, transferrin and the third component of complement) was monitored in the supernatants from culturing cell hybrids, between normal diploid rat fibroblasts and mouse hepatoma as well as between normal rat liver cells and mouse fibroblasts of the permanent line A9 [78]. A saponin isolated from *Anemarrhena asphodeloides* Bunge was found to reduce by 50% α-fetoprotein (α FP) production when injected into newborn rats by acting on α FP gene expression through a glucocorticoid receptor-mediated action [79]. Rats given the antithyroid drug propylthiouracil showed a 30% reduction in the lytic activity of the classical complement pathway, and an 80% increase in activity of the alternative complement pathway; the latter effect subsided when prolonging treatment up to 30 days [80].

A few reports on varying serum protein levels have dealt with toxicological studies. Some addressed the behaviour of individual proteins. Serum albumin was monitored on biliary fistula rats and on isolated perfused rat livers to assess the inhibition by sodium valproate (an antiepilectic drug) on the movement of secretory vesicles in rat hepatocytes [81].

In some reports in which 2-DE was applied, the protein spots were marked by numbers, not associ-

ated with names. Marshall and Vesterberg could only tentatively identify orosomucoid among the potential marker proteins for exposure to carbon tetrachloride, trichloroethylene or dimethylformamide [82]. No identification was provided by Zastrow et al. for the 12 spots (among 410 resolved components) up- or down-regulated by exposure to isobuthylacetate and/or methoxyethylacetate [83].

5. Rat serum proteins: pharmacological studies

5.1. Testing strategies

Selection of a useful experimental model for in vivo drug testing must aim at clear (statistically highly significant) differences between test and control animals in readily measured parameters. In this respect 2-DE of rat serum offers a number of advantages.

Blood drawing is a minimally invasive procedure that may be repeated on the same animals while causing only minor distress. This makes it especially suited for time-course experiments. In a general sense, repeated sampling allows the same animals to be dealt with: (i) as controls at time zero, without treatment; (ii) as disease models after noxious treatment; and/or (iii) as test targets after drug treatment. Such a protocol allows to optimise data reliability (test potency), i.e. to account for inter-individual biological variation while using the smallest number of animals. The latter point is most relevant in several respects. Concern about the use and the sacrifice of living animals is growing both in the scientific community and in the general population; the authors thoroughly support this ethical issue. It is and it will be impossible to do without in vivo experiments, specifically in vivo drug testing, as the only ones able to account for systemic reactions and overall metabolism. Reduction in the numerosity of the test groups on the contrary is an achievable goal that should be sensibly addressed and tenaciously pursued. Likewise, costs for animal care have drastically increased in connection with improvements in housing facilities and strengthening of animal handling rules. Saving money without impairing the quality of the collected data is then a most desirable result. Moreover, defining protocols based on serum analysis already at the stage of pharmacological investigation offers a substantial advantage when clinical trials begin on human patients, and the sample of choice, most often the only expedient biological sample, is indeed serum.

2-DE is a comparatively demanding technique; however, it is so much more performing than any other approach for serum protein analysis that on a cost/benefit basis it outweighs all alternative techniques. Moreover, state-of-the-art protocols, equipment, reagents and consumables allow for even inexperienced colleagues to run good quality gels after little practising. A time-consuming, labour-intensive step in the whole procedure is spot quantification. This is possibly one of the reasons why not all reports on the structural aspects of proteomics for a given specimen (identification of all major component peptides) are followed by further reports on the functional aspects of proteomics (changes in individual protein expression as a function of the experimental conditions). Rigorous quantification and adequate statistical analysis on numerical data are indeed required for any worthy assessment of experimental results. However, for a rough evaluation of the trends, it is very favourable that the baseline concentration in rat serum of some APPs (α_1-MAP, α_1-AG, Hp) is zero. The percent change when an inflammatory condition does develop is then obvious even by simple visual inspection. In comparison with other mammalian sera (from man, mouse, cow—our results, some of which unpublished), rat serum scores as a sample easily amenable to thorough resolution and quantitative analysis. This outcome is made possible by an even abundance of the various proteins (with a comparatively low albumin content) and by an even pI/M_r distribution (without extensive overlaps). It should be recalled, however, that serum proteins are almost invariably (micro)heterogeneous as a result of post-translational modifications, including deamidation (apoA-I), phosphorylation (α_2-HS) and, more commonly, glycosylation. This implies that individual spots must be referred to identified proteins and the contributions of all relevant components taken into account by summing them up. This requirement may be contrasted to the features of most intracellular proteins, for which post-translational modifications are a much less common occurrence. 2-DE allows at the

same time overall quantification (summing up spots) and detection of PTMs/binding behaviour (e.g. Fig. 5) which result in positional shifts of the spots.

After an accurate definition of the serum protein pattern under ''control'' and ''diseased'' conditions, two sets of test treatments should be performed on rats in the actual pharmacological tests, and sera from the corresponding animals should be analysed. The first such set refers to the administration—in varying amounts, or according to different schedules, or through different ways—of the test drug to ''diseased'' animals. The criterion according to which a successful treatment may be scored is the percent recovery towards ''control'' conditions for the levels of those proteins whose expression is altered in the ''diseased'' conditions. The second, and not less relevant, set of treatments should refer to the administration of the test drug to ''control'' animals. While the first set is obviously meant to assess the effectiveness of the test drug, the second tries to evaluate unwanted reactions and side effects. Our experience with anti-inflammatory drugs shows 2-DE analysis of rat serum to be more sensitive (as wider in scope) than individual biological assays. It is worth reminding that 2-DE inherently evaluates—in principle—all proteins in a biological sample (in practice, it evaluates all major spots, depending on the resolution afforded by the selected protocol and on the sensitivity of the selected staining procedure). This amounts in rat serum to tens of proteins resolved in hundreds of spots. With 2-DE any change in these components is open to inspection, hence to ''discovery''. Although in this review up/down-regulation and quantitative aspects will be the only aspects on focus, our experience with rat body fluids (serum itself, urine, CSF) has allowed a number of observations, some of which still to be published, on diverse phenomena. For proteins present at such a low concentration under baseline condition that their identification had not been possible with standard running conditions and protein loads, pathological states or pharmacological treatments were found after which their expression was highly increased. Such observations raised the interest for the characterisation of these proteins as well as made it possible to gain enough material for analysis by mere spot excision from standard gels. Structural analysis by MS techniques allowed the recognition of a novel primary sequence, the definition of the proteolysis site in a protein precursor, the identification of previously unreported post-translational modifications in specific proteins [6]. Changes in isoform assortment for serum glycoproteins, differential expression of isoforms in various biological fluids or differential transport between different compartments was also observed (Fig. 5). It is possible—although neither necessary nor recommended—to work with 2-DE without having to prove/disprove a specific working hypothesis and without having selected one/few targets. This situation is sometimes resented as less scientifically sound than its customary alternative, i.e. thoroughly testing ''about'' one/few marker(s) at a time. We agree that a list of affected proteins is the first step towards understanding; this means the most remote from the goal but also the closest achievement, the most needed one, along the path to knowledge.

Literature data on rat serum proteins in connection with drug testing is unevenly categorised, depending on the topic on focus:

- studies on the (non-covalent) interactions between chemicals and (carrier) proteins, and on competition between ligands
- studies on the covalent reactions between chemicals and protein reactive groups
- studies addressing the changes in serum concentration of lipoproteins/apolipoproteins upon administration of hypo-lipidemic drugs
- studies addressing changes in APP levels after administration of anti-inflammatory drugs

5.2. Non-covalent drug–protein interactions

This series of reports usually addresses the issue of binding to α_1-AG for cationic drugs, or to SA and its foetal counterpart α-FP for lipophilic/anionic drugs. For instance, an increased propranolol binding to rat serum was found to be induced by phenobarbital administration, most likely as a result of increased concentrations of α_1-AG [84]. The observation that Dark Agouti rats appeared more sensitive to phenobarbital vs. Sprague–Dawley suggests that this strain should be used preferentially for the study of the effect of cytochrome P-450 inducers on α_1-AG

metabolism and cationic drug binding [84]. Among quinolonecarboxylic acids, cinoxain and nalidixic acid bound to albumin whereas pipemidic acid showed a weak interaction with apotransferrin [85,86]. Quinolones (anti-microbial agents) on the contrary bound mainly to albumin; their affinities varied with an inverse relationship to the size of the substituent at the 7-position of the molecule, possibly due to a steric effect [87]. Coadministration of fenbufen and ciprofloxacin did not affect binding to serum proteins; a significantly prolonged plasma half-life of the drug was due to inhibition of its renal excretion [88]. Selenite treatment caused a reduction in binding capacity of serum proteins to Hg^{2+} and phenylmercuric acetate [89].

Varying degrees of affinity and capacity influence pharmacokinetic parameters and protein binding may result either in a reservoir or in a sink for the dosed drug. For instance, rat serum had a large binding capacity for phencyclidine but the affinity was low and the binding readily reversible [90]. Conversely, in vitro 195mPt-*cis*-dichlorodiammine platinum(II) bound essentially irreversibly to plasma proteins [91].

Interference by endogenous compounds on serum protein binding is also on focus in these reports. Binding of various compounds, including phenytoin and salicylic acid, was found to be significantly decreased in uremic rat plasma. This effect was likely caused by the presence of endogenous binding inhibitors that could be removed by charcoal treatment [92]. Competition for the binding to SA is often by free fatty acids (FFA). The half-life of *p*-chlorophenoxyisobutyrate, an active metabolite of clofibrate and a compound extensively bound to plasma proteins, decreased significantly in the presence of high plasma FFA levels [93]. A good correlation was observed between the dissociation constants of cefazolin binding and the molar ratio of FFA to SA concentration in sera [94]. In genetically obese Zucker rats the mean unbound fraction for various sulfonilamides was significantly higher than in lean rat serum as a result of elevated FFA concentrations [95].

Techniques used in these investigations are typically dialysis and ultrafiltration, but also high-performance displacement chromatography [96] or high-performance frontal analysis with a chiral separation column [97].

Modes of drug binding and features of the protein binding sites are often defined through spectroscopic procedures (circular dichroism, fluorescence quenching). For instance, the effect of phenylbutazone binding on the intrinsic fluorescence of rat αFP indicated that its tryptophan residue is not in the same molecular environment as that of albumin, and does not participate directly in the high-affinity site for phenylbutazone [98]. Chromatography on stationary phases based on immobilized albumins demonstrated that the binding sites on rat SA are different from those in human (or rabbit) SA; for the chiral compounds studied, the stereoselectivity of drug binding appeared much lower in rat than in human SA [96]. In binding competition experiments, αFP showed high-affinity binding for the specific markers (warfarin, phenylbutazone, azapropazone) of albumin's drug-binding site I whereas it had neither albumin's high-affinity site II (diazepam as marker) nor its site III (digitoxin and cholic acid as markers) [99]. However, in a case of toxicological relevance, immunoelectrophoresis and autoradiography were used to study the transport of $chromium^{3+}$ by rat serum proteins [100].

5.3. Covalent drug–protein reactions

For some drugs irreversible binding is part of their action mechanism (e.g. iproniazid and selegiline, neostigmine and eserine, sulphonamides, penicillins, clavulanic acid, chloramphenicol, aspirin, disulfiram, azaserine and 5-fluorouracil, 4-hydroxyandrostendione). However, this effect is to occur with high selectivity at one target site (one amino acid) in one target cellular component, often a receptor or an enzyme. Unspecific adduct formation with serum proteins is on the contrary regarded as an unwanted side effect. This covalent binding alters pharmacokinetic parameters. For instance, [^{3}H]17 α-ethinylestradiol was found to irreversibly bind to proteins; after chronic administration the radioactivity accumulated in plasma, lung, brain, kidney, spleen and liver (the soluble cell fraction of the latter being labelled more than the microsomal fraction) [101].

A beneficial effect of unspecific protein reaction may be described only as preventive of noxious interactions on the same targets. For instance, phosphorothioate drugs act as radioprotective agents by

forming mixed disulfides with proteins of the liver, kidney, lung, brain, and serum [102].

Most dangerously, the covalent reaction products between proteins and drugs may even bring about noxious reactions when eliciting immune responses. This is for instance the case with the acyl glucuronide metabolites of carboxylic acid drugs, such as the salicylate derivative diflunisal. Their covalent adducts with serum proteins lead to the production of circulating antibodies which may be involved in aberrant immune responses such as drug hypersensitivity [103].

Reaction between serum proteins and penicillins had been detected by 2-DE mapping of specimens from human patients under antibacterial therapy [104,105]. Other examples of drugs or drug metabolites known to bring about non-enzymatic covalent post-translational modifications of proteins are listed in [106].

Still on the noxious side, covalent adduct formation may mediate toxicological effects. After administration of the carcinogen [2,2′-3H]-4-aminobiphenyl some metabolites (4-aminobiphenyl, 4′-hydroxy-4-acetylaminobiphenyl) were found to bind non-covalently to albumin, whereas 3-tryptophanyl-4-acetylaminobiphenyl formed by reaction with the unique tryptophan at the high-affinity binding site [107]. Covalent protein modifications had been detected by 2-DE in rat liver following administration of methapyrilene [108].

A different perspective is with the use of serum proteins as carriers. This may involve reaction of SA with a peptide component. For instance, recombinant granulocyte-colony stimulating factor was covalently conjugated to SA through a heterobifunctional maleimido–carboxyl polyethylene glycol; clearance rate was reduced and stability increased [109]. Otherwise, SA may be radioiodinated according to different protocols. Using sequential scintigraphy, SA labelled at a residualizing marker tagged to it was shown to be taken up and metabolised in tumors better than after direct radioiodination [110].

5.4. Hypo-lipidemic drugs

In clofibrate-treated normolipemic rats the concentrations of serum apolipoproteins A-I, B and C-III were reduced, whereas the apolipoprotein E level was not altered. The distribution of apolipoproteins A-I, B, C-III and E between heparin-Mn supernatant and precipitate were unaffected. The unchanged C-III distribution indicates unaltered intravascular VLDL catabolism. Concurrent reductions in serum HDL cholesterol and apoA-I in clofibrate-treated rats suggest a diminished production of lipoprotein particles containing apoA-I. Reductions in serum apoB and in the mass ratio of serum triglyceride to apoB indicate a decrease in the number and size, respectively, of circulating triglyceride-rich lipoprotein particles. These observations suggest that the hypolipidemic effect of clofibrate in the normolipemic rat is caused mainly by diminished hepatic secretion, rather than by enhanced catabolism, of triglyceride-rich lipoproteins [111].

After hypophysectomy in female rats, there occurred a decrease in serum cholesterol and serum levels of apo A-I and apo E, in spite of replacement therapy with T4 and cortisone. Similar changes were also observed in HDL. In contrast, apo B, cholesterol, and triglycerides were increased in LDL. Estradiol treatment had no effect on these changes. Continuous infusion of growth hormone (GH) resulted in an increase in cholesterol and apo E in serum and HDL to the levels of intact females. In contrast, GH given twice daily had no effect. Therefore, the sexually dimorphic secretion of GH may be important for the regulation of sex differences in apo E and HDL cholesterol levels. There were no consistent effects of GH treatment on the levels of apo A-I in serum or HDL, but GH treatment resulted in a decrease in apoB and triglycerides in both serum and LDL, regardless of the mode of administration. This suggests that GH regulates the serum and LDL levels of apo B and triglycerides independently of the secretory pattern [112].

5.5. Anti-inflammatory drugs

Very few research groups have made reference so far to APPs when assessing the activity of anti-inflammatory drugs; they used such methodological approaches as automated quantification of some proteins with a centrifugal analyzer [113] and lectin affinity blotting of ConA-reactive proteins [114]. Two substituted heterocyclic alkoxypropionic acids, romazarit [115] and bindarit [114], were tested as potential antirheumatic agents along these lines. In

an extensive comparison of established drugs for the treatment of arthritis, after induction of (acute) inflammation by adjuvant injection, rats received medication for 5 days [116]. Before drug administration, APP levels correlated with paw swelling. NSAIDs reduced the swelling and raised levels of Hp and Cp in a dose-related manner. Steroids at high dose also reduced swelling, lowering α_1-AG, Hp, Cp and raising SA, whereas at low doses they attenuated paw swelling but only affected SA concentration. Antirheumatic drugs lowered the levels of α_1-AG, Hp and Cp without reducing swelling.

In our investigation on the effects of non-steroidal anti-inflammatory drugs (NSAIDs) we closely complied with the guidelines given at the beginning of this section (Section 5.1). The test pathological state was adjuvant arthritis, a condition taken as an animal model of human rheumatoid arthritis. Lewis female rats were assigned into six experimental groups: (i) control animals (C); (ii) animals with adjuvant arthritis (AA); (iii) animals without AA treated with indomethacine (Ind); (iv) animals without AA treated with ibuprofen (Ibu); (v) animals with AA treated with indomethacine (AIn); and (vi) animals with AA treated with ibuprofen (AIb). AA induction was through immunisation with *M. tuberculosis* (Section 3); treatment with NSAIDs started on day 3 after AA induction, testing was on day 14. Drug doses were: for indomethacine (water-soluble salt), 1.5 mg/kg s.c. twice daily; and for ibuprofen (lysine salt), 25 mg/kg s.c. twice daily. The selected indomethacine dose is known to cause neither general nor local adverse effects (experimentally no gastrointestinal lesions) in rats. It is also close to therapeutic doses in humans, typically 1–2 mg/kg per os daily. Rat serum proteins were quantified after 2-DE analysis.

AA vs. C comparison is part of Fig. 4 (solid bars), in which percent changes in protein levels are reported for the experimental condition in respect to baseline (>500% increase refers to those APPs whose concentration in serum of control animals is below detection limit, and implies de novo synthesis).

AIn vs. AA and AIb vs. AA are shown in Fig. 7 which thus depicts the efficacy of the two drugs, at the stated doses, in inhibiting the effects of arthritis on the levels of rat serum proteins. A high variability

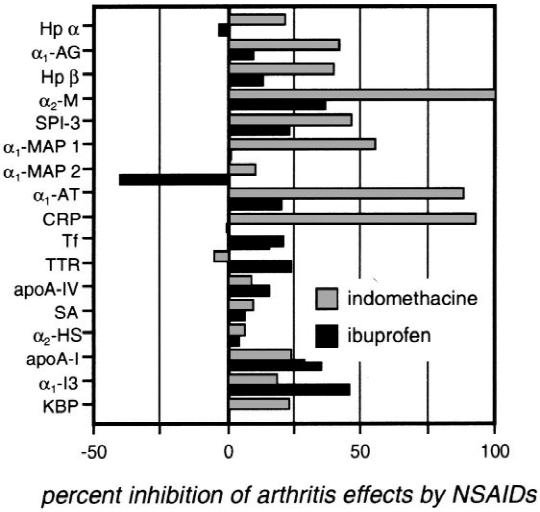

Fig. 7. Assessment of adjuvant arthritis treatment. Efficacy of NSAIDs given to affected rats (indomethacine, light grey filling; ibuprofen, dark grey filling) was evaluated as restoration of serum proteins levels close to their control values [9]. Data are computed as: 100*(AA − AIn)/(AA − C) for indomethacine and as 100*(AA − AIb)/(AA − C) for ibuprofen, i.e. changes due to treatment (comparison treated/non-treated) are referred to changes due to arthritis (comparison healthy/arthritic rats).

of responses is observed, for various proteins to each NSAID and for the same protein between NSAIDs. α_2-M is most responsive in AIn, α_1-I3 in AIb. However, on the average, the inhibitory effects are two to three times as large for indomethacine as for ibuprofen. These findings closely correlate with therapeutic data. In the same animals, indomethacine was able to restore a growth curve closer to normal, as well as it was inhibiting paw swelling to a larger extent than ibuprofen [9].

NSAIDs, while treating the inflammatory pathology, paradoxically up-regulate the levels of circulating pro-inflammatory cytokines such as TNF [117–119] and IL-6 [120]. This is because prostaglandins (in particular PGE2) are important negative regulators of TNF production [121]. However, this effect depends on the drug used, the target organ and the experimental model [122–124]. Interestingly, no such up-regulation is generally seen for the APPs: only the levels of α_1-MAP(2) are raised in AIb more than in AA; in all the other cases, the effect of AA is

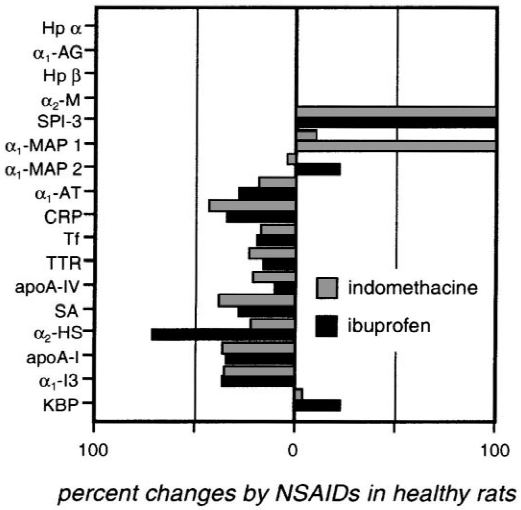

Fig. 8. Assessment of unwanted drug effects. Severity of side effects was evaluated as changes from baseline levels of serum proteins in healthy rats treated with NSAIDs (indomethacine, light grey filling; ibuprofen, dark grey filling) [9]. Data are computed as: 100*(Ind − C)/C for indomethacine and as 100*(Ibu − C)/C for ibuprofen.

inhibited to a variable extent by indomethacine and ibuprofen.

Fig. 8 compares Ind vs. C and Ibu vs. C. NSAIDs given to rats without AA reproduce some of the effects of inflammation. Ibuprofen, which is less effective than indomethacine in inhibiting inflammation and APP alterations induced by AA, is more potent in producing ''pseudo-inflammatory'' effects per se.

The mechanisms underlying these effects are unclear. The acute phase response is triggered by the activation of stress-sensitive kinases (ERK, p38, JNK) that phosphorylate transcription factors belonging primarily to C/EBP, AP-1 and NF-κB families. These activate the gene promoters of acute phase cytokines and regulate APPs, directly or through the action of the cytokines themselves [125]. IL-6 is a major activator of APPs [126–128], and is a good candidate for the pseudo-inflammatory effects of NSAIDs, which, in fact, induce IL-6 in some situations. For instance, indomethacine and ibuprofen increased serum IL-6 activity in mice [117]. Moreover, naproxen induces IL-6 in human peripheral blood mononuclear cells [120] and prostanoids induce IL-6 in mast cells [129]. However, our experimental model does not confirm this, since serum IL-6 activity after NSAID administration was below detection limit [9].

Another factor in the APP cascade is NF-κB, which activates several APPs, including serum amyloid A [130], α_1-antichymotrypsin [131] and α_1-AG [132]. Some NSAIDs (notably aspirin and salicylates) inhibit NF-κB activation [133,134]. Indomethacine and ibuprofen, used in the same investigations as reference compounds, did not inhibit NF-κB activation by LPS and—although the question was not directly addressed by the authors—in one report [134] they seemed to enhance this activation. Additionally we have evidence that indomethacine per se can induce NF-κB activation in mouse peritoneal macrophages (unpublished observation).

We suggest therefore (Fig. 9) that NF-κB activation might be one of the mechanisms of APP induction by indomethacine and ibuprofen. In addition, NSAID treatment per se is capable of inducing TNF (which is induced by, and activates NF-κB). This effect is thought to be associated with gastroin-

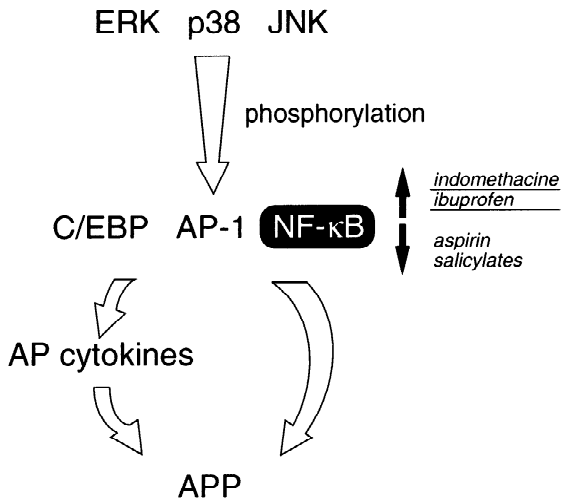

Fig. 9. The acute-phase response cascade. APP synthesis may be turned on either (left arrow) by acute-phase cytokines (up-regulated themselves by various transcription factors) or (right arrow) directly by NF-κB [9].

testinal toxicity, and has been extensively studied with indomethacine [135–137].

6. Conclusions

So far rat serum was not a commonly analysed sample in pharmacological and toxicological research. We believe our work on rat serum and more recently on rat urine and cerebrospinal fluid has established useful databases with identification/characterisation of all major protein components. Our own data on NSAIDs prove that a correlation exists between biochemical and therapeutic indexes. From the same data it seems inappropriate to select any single protein as a privileged marker of the efficacy of a given drug. On the contrary the quantification of a panel of serum components, as allowed by 2-DE, permits to assess both desired therapeutic and undesired collateral or even toxic effects.

7. Nomenclature

α_1-AG	α_1-acid glycoprotein, or orosomucoid
α_1-AT	α_1-antitrypsin
αFP	α-fetoprotein
α_1-I_3	α_1-inhibitor III
α_1-M	α_1-macroglobulin
α_1-MAP	α_1-major acute phase protein, or thiostatin
α_2-HS	α_2-HS-glycoprotein
α_2-M	α_2-macroglobulin
AA	adjuvant arthritis
AI	acute inflammation
AIb	treatment of arthritis rats with ibuprofen
AIn	treatment of artritis rats with indomethacine
apo	apolipoprotein
APP	acute phase protein
CD	Sprague–Dawley rats
CI	chronic inflammation
Cp	ceruloplasmin
CRP	C-reactive protein
Gc	Gc-globulin
GH	growth hormone
HDL	high-density lipoprotein
Hp	haptoglobin
Ibu	treatment of control rats with ibuprofen
IL	interleukin
i.m.	intramuscular
Ind	treatment of control rats with indomethacine
KBP	kallikrein-binding protein
LCAT	lecithin cholesterol acyltransferase
LDL	low-density lipoprotein
LW	Lewis rats
NF-kB	nuclear factor κ B
NSAID	non steroidal anti-inflammatory drug
PAA	polyacrylamide
SA	serum albumin
s.c.	subcutaneously
SPI3	serine protease inhibitor-3
Tf	transferrin
TNF	tumor necrosis factor
TTR	transthyretin
VLDL	very low-density lipoprotein

Acknowledgements

Work supported in part by FIRB grants from Università degli Studi di Milano to EG.

Appendix A. Our protocol for 2-DE analysis of rat serum

Gel format: Our typical 2-DE gel size is 16×14 cm, corresponding to the format of the Protean II™ vertical chamber (BioRad, Hercules, CA). For faster screening, to each 2d slab we may apply, end to tail, two strips from 1d run, 8 cm anode-to-cathode distance.

Sample: Plasma should not be substituted for serum; its stability upon storage is uneven, and somehow unpredictable. Small to large clots are often observed upon freezing–thawing, which trap variable amounts of different proteins. This makes impracticable any quantitative evaluation of plasma proteome.

We make up samples by simple dilution of serum with 2% 2-mercaptoethanol and incubation at room temperature (r.t.) for 15–30 min. To 16 cm long IPG we apply 15 μl of a dilution of 15 μl of serum with

35 μl of 2% 2-mercaptoethanol (which corresponds to 4.5 μl undiluted serum). To 8 cm long IPG we apply 5 μl of a dilution of 4 μl of serum with 6 μl of 2% 2-mercaptoethanol (which corresponds to 2 μl undiluted serum). Under these conditions, the Coomassie stained spot of albumin is always saturated, that of Tf is usually not and is amenable to densitometric evaluation. (Albumin may be determined spectrophotometrically by the immediate bromocresol green assay [183,184].) For purposes other than quantification of individual components we could load at least 10 times more protein, i.e. 40 μl serum. The outlined treatment is unable to unfold fully iron saturated Tf (Fig. 5). This is a minor nuisance, as it increases the number of spots (isoforms) whose contribution has to be summed up to account for Tf amount/concentration. However, the ratio between folded and unfolded Tf (acidic vs. basic spots) could be taken as an index of iron status in the test animals. From our experience [185], we are unconvinced that reduction/alkylation (carboxymethylation) is a worthwhile procedure for sample preparation: although it often appears to increase the amount of protein able to focus during the 1d run, it seems also to multiply (artefactually?) the heterogeneity of the protein pattern. We have no evidence of mixed disulfide formation during 1d run; some problems about re-oxydation during 2d runs are discussed below. The opposite view is discussed, and detailed protocols for protein carboxymethylation prior to 2DE analysis are given in Refs. [186–188].

IPG: We polymerise our own gels according to the procedures in Ref. [3], either the same day they are to be used for 1d runs or the day before. Gel composition is given in Appendix A, Table 1, for the most commonly used gradient, pH 4–10 with a non-linear (exponential) course [1]. For narrow ranges, we usually interpolate the gel composition from the recipe for the wider gradient (see Ref. [3]); for the resolution of either α_1-AG/clusterin or apoC we make use of IPG pH 2.5–5 [2].

Two options are open for shaping and reswelling the individual IPG strips on which the various samples are run during the focusing step.

According to the first protocol, along with the guidelines for ready-made gels, the dry slabs (gel and plastic) are cut into 4 mm-wide strips. These are aligned in the desired number within a reswelling chamber taking care their back adheres to the supporting glass while their front (the gel side) faces the silanized molding plate. An extra gasket cut from Gel Bond and overlaid to the permanent rubber frame allows the required thickness to be exacly matched and leaks to be avoided. The reswelling solution is added slowly from the lower port with a syringe. These narrow strips should then be run with appropriate protection towards drying (paraffin oil).

The approach [189] we still prefer uses 7 mm-wide strips of gels cut on an intact plastic support. In this case, the reswelling cassette is assembled around the whole slab.

Trapping air bubbles is avoided by pumping the solution evenly from the bottom (according to the manufacturer's instructions) or by letting it flow along the edges, if the mold is filled from the top. The gel begins reswelling right away, still it is possible within few minutes to draw and add back some of the liquid trying to force air bubbles to float. It is also possible, once the mold is full, to remove, or just push aside, the most tenacious bubbles with a hook cut from GelBond foil.

We reswell the gels for rat serum analysis 1–5 h in 8 *M* urea/0.5% w/v CA (see Appendix B, Table 2).

Procedure for 1d run: According to our protocol [189], gel is removed, anode to cathode, by cutting through with a long, straight knife, then sliding it against the plastic support, to leave 7 mm-wide strips for sample application. Eight such strips, 4 mm apart, are typically obtained from a 16 cm long slab. It is advised to leave wide gel strips along the edges, in order to prevent drying of the sample lanes. Shreds of gel not removed by the knife are scraped with a straight scalpel blade.

The individual lanes are marked on the back with a felt tip for sample identification. The slab is aligned on the cooling plate: a couple of milliliters of distilled water allow a thorough contact with the surface. Paper tissues are lined to the anodic and cathodic edges to collect excess liquid also throughout the following run (e.g. overflow of non-isoelectric CAs from narrow pH rage IPGs). Electrode strips moistened in distilled water are overlaid to the gel. In this protocol, samples are usually applied with the help of Paratex pads. One such pad may hold up to 25 μl; up to four layers may be overlaid. Serum is applied to the IPG strip near the cathode. Indeed, SA

migrates poorly if applied to the IPG at a pH lower than its p*I* [189]; streaked patterns usually result from direct reswelling of the gel strips in a serum-containing solution.

Our selection of IPG running conditions is very conservative in comparison with current protocols [190]. The initial voltage we use is 100 V for an electrode distance of 8 cm, of 250 V for an electrode distance of 16 cm and a wide pH range, of 400 V for an electrode distance of 16 cm and a narrow pH range. The settings for the overnight run (13–14 h) are 400, 700 and 1200 V, respectively. These conditions are arrived at by a stepwise increase over approximately 5 h—which means the run is started in the early afternoon. The next day the bands are sharpened for 2 h at 1200 V for the 8 cm distance and at 3500 V for the 16 cm distance. The temperature of the cooling plate is 15 °C.

SDS–PAGE: We run our 2d on 7.5–17.5% T PAA; gel composition for individual slabs is given in Appendix B, Table 3. To the purpose of identification, we mark each SDS slab with a number, pencilled or typed on a square of chromatographic paper. To increase resolution between α_1-MAP/KBP/α_2-HS/SPI-3/α_1-AT, softer PAA may be used, e.g. a 4.5–9% T PAA.

The SDS–PAGE slabs for 2d are usually polymerised the day before and stored overnight in a cold room, under a layer of water–buffer and covered with cling film. They are then allowed to warm to room temperature before use, while any liquid is removed by inverting on a towel.

1d–2d equilibration and 2d run: Paratex pads and electrode strips are removed from IPG surface after moistening with a stream of distilled water.

Incubation is usually for 15 min at room temperature, on a shaking platform, in 50–70 ml of electrode buffer (Tris–glycine according to Laemmli [191]) added with 3% w/v SDS, 2% v/v 2-mercaptoethanol and 1% v/v saturated bromophenol blue solution. With this protocol Hp α chains appear to migrate as dimers, giving rise to somehow diffuse spots; this problem may be circumvented by resorting to the two-step protocol of alkylation–reduction [192].

The lanes corresponding to individual samples are trimmed by cutting the plastic support flush to one side and approximately 2 mm away from the other. At this stage they may be frozen by laying on dry ice (over a plastic bag), then stored at −20 °C for further use; ice (buffer) should not be allowed to thaw before interfacing to the 2d slab.

A sol of 0.7% w/v low electroendosmosis agarose in electrode buffer is prepared about 30 min in advance by boiling the suspension in a water bath. The sol is maintained at 60–65 °C either by transferring the flask to a thermostatted bath or by lowering the setting of the heating plate.

Agarose sol is poured in the rim above the SDS–PAGE gel; the individual sample strips are inserted promptly in the warm sol, flush side down, and aligned with a flat spatula. The IPG strip is oriented anode (lower p*I*) to the left and cathode (higher p*I*) to the right. Once the agarose is set, the SDS–PAGE slab with its overlay is moved to the vertical electrophoretic chamber for the 2d run.

The settings for SDS–PAGE are 50–70 mA/slab, 450 V and 60 W maximum per cell; the run lasts approximately 3 h.

Staining and imaging: Gels are stained with 0.3% w/v Coomassie R in 30% v/v ethanol:10% v/v acetic acid. After destaining the gels belonging to the same batch are stored more than 24 h in 10% v/v acetic acid to achieve similar background and spot intensity.

The 2-DE gels are scanned with a video camera (Sony, Japan) under the control of NIH Image, and analysed with the software PDQUEST (PDI, Huntington Station, NY).

Quantification and data reduction: Spot detection is performed after background correction in the gel images, followed by manual editing of all gels in one series (usually one run). A matchset is created including all gels to be compared. A standard gel (master) is generated out of the image with the highest spot number by including additional spots from the other gels [193]. As all gels within one series are processed ''in one batch'', results are evaluated in terms of spot volumes without further correction/normalization. For those proteins which consist of a spot train, single spot volumes are summed up. Nevertheless, the overall pattern/position of the spot chain is inspected, to determine positional shift of proteins, being a sign, e.g. for post-translational modifications.

Appendix B. Gel composition

Table 1. Isolectric focusing non-linear pH 4–10 IPG

	pH 4	pH 10
pK 0.8 (μl)	138	16
pK 4.6 (μl)	130	–
pK 6.2 (μl)	153	46
pK 7.0 (μl)	–	61
pK 8.5 (μl)	–	23
pK 9.3 (μl)	–	43
1 *M* AcOH (μl)	–	14
T30 C4[a] (μl)	680	680
Glycerol (μl)	1000	–
TEMED (μl)	3.1	2.7
d.d. water to (ml)	5.1	5.1
40% APS (μl)	4.75	4.75

[a] Acrylamide stock, containing 28.8 g acrylamide and 1.2 g bis/100 ml.

Table 2. Composition of the CA mix for IPG reswelling

4–6.5 Pharmalyte	1.2 ml
4–6 Ampholine	1.2 ml
5–7 Ampholine	1.6 ml
3–10 Pharmalyte	2.0 ml
3.5–10 Ampholine	2.0 ml

Table 3. SDS electrophoresis

	17.5% T	7.5% T	3% T PAA[a]
T30C4 (ml)	9.5	4.1	1
Running buffer 5×stock (ml)	3.3	3.3	
Stacking buffer 5×stock (ml)			2
SDS 4% (μl)	410	410	250
BPB (μl)	115	–	
Glycerol to (ml)	16.5	–	
d.d. water to (ml)	–	16.5	10
TEMED (μl)	8.2	8.2	5
40% APS (μl)	12.5	25	15

[a] 3.5 ml per slab, add APS after degassing.

References

[1] E. Gianazza, P. Giacon, B. Sahlin, P.G. Righetti, Electrophoresis 6 (1985) 53.
[2] E. Gianazza, F. Celentano, C. Ettori, P.G. Righetti, Electrophoresis 10 (1989) 806.
[3] E. Gianazza, in: A.J. Link (Ed.), 2-D Proteome Analysis Protocols, Humana Press, Totowa, 1998, p. 175.
[4] P. Haynes, I. Miller, R. Aebersold, M. Gemeiner, I. Eberini, M.R. Lovati, C. Manzoni, M. Vignati, E. Gianazza, Electrophoresis 19 (1998) 1484.
[5] I. Miller, P. Haynes, M. Gemeiner, R. Aebersold, C. Manzoni, M.R. Lovati, M. Vignati, I. Eberini, E. Gianazza, Electrophoresis 19 (1998) 1493.
[6] R. Wait, E. Gianazza, I. Eberini, L. Sironi, M.J. Dunn, M. Gemeiner, I. Miller, Electrophoresis 22 (2001) 3043.
[7] I. Miller, P. Haynes, I. Eberini, M. Gemeiner, R. Aebersold, E. Gianazza, Electrophoresis 20 (1999) 836.
[8] I. Eberini, I. Miller, V. Zancan, C. Bolego, L. Puglisi, M. Gemeiner, E. Gianazza, Electrophoresis 20 (1999) 846.
[9] I. Eberini, D. Agnello, I. Miller, P. Villa, M. Fratelli, P. Ghezzi, M. Gemeiner, J.H. Chan, R. Aebersold, E. Gianazza, Electrophoresis 21 (2000) 2170.
[10] L. Sironi, E. Tremoli, I. Miller, U. Guerrini, A.M. Calvio, I. Eberini, M. Gemeiner, M. Asdente, R. Paoletti, E. Gianazza, Stroke 32 (2001) 753.
[11] I. Eberini, I. Miller, G. Gemeiner, P. Haynes, R. Aebersold, L. Puglisi, C.R. Sirtori, E. Gianazza, Electrophoresis 20 (1999) 3599.
[12] F. Gauthier, K. Ohlsson, Hoppe-Seylers Z. Physiol. Chem. 359 (1978) 987.
[13] K. Lonberg-Holm, D.L. Reed, R.C. Roberts, R.R. Hebert, M.C. Hillman, R.M. Kutney, J. Biol. Chem. 262 (1987) 438.
[14] T.A. Braciak, W. Northemann, G.O. Hudson, B.R. Shiels, M.R. Gehring, G.H. Fey, J. Biol. Chem. 263 (1988) 3999.
[15] C. Gardmo, B. Persson, A. Mode, Endocrinology 142 (2001) 2695.
[16] L. Belanger, S. Roy, D. Allard, J. Biol. Chem. 269 (1994) 5481.
[17] G. Schreiber, H. Dryburgh, A. Millership, Y. Matsuda, A. Inglis, J. Phillips, K. Edwards, J. Maggs, J. Biol. Chem. 254 (1979) 12013.
[18] J.I. Huggenvik, R.L. Idzerda, L. Haywood, D.C. Lee, G.S. McKnight, M.D. Griswold, Endocrinology 120 (1987) 332.
[19] S. Welch, A. Skinner, Comp. Biochem. Physiol. 93B (1989) 417.
[20] G. Schreiber, A. Tsykin, A.R. Aldred, T. Thomas, W.-P. Fung, P.W. Dickson, T. Cole, H. Birch, F.A. De Jong, J. Milland, Ann. N.Y. Acad. Sci. 557 (1989) 61.
[21] M.W. Heiny, N. Takahashi, S. Noda, F.W. Putnam, Prot. Biol. Fluids 34 (1986) 431.
[22] D. Wellner, K.C. Cheng, U. Mueller-Eberhard, Biochem. Biophys. Res. Commun. 155 (1988) 622.
[23] H. Nikkila, J.D. Gitlin, U. Mueller-Eberhard, Biochemistry 30 (1991) 823.
[24] T.D. Sargent, M. Yang, J. Bonner, Proc. Natl. Acad. Sci. USA 78 (1981) 243.
[25] T.D. Sargent, L.L. Jagodzinski, M. Yang, J. Bonner, Mol. Cell Biol. 1 (1981) 871.
[26] G. Rauth, O. Pöschke, E. Fink, M. Eulitz, S. Tippmer, M. Kellerer, H.-U. Häring, P. Nawratil, M. Haasemann, W. Jahnen-Dechent, W. Müller-Esterl, Eur. J. Biochem. 204 (1992) 523.
[27] E. Olivier, E. Soury, P. Ruminy, A. Husson, F. Parmentier, M. Daveau, J.-P. Salier, Biochem. J. 350 (2000) 589.
[28] J. Chao, K.X. Chai, W. Xiong, S. Chao, C. Woodley-Miller, L. Wang, H.S. Lu, L. Chao, J. Biol. Chem. 265 (1990) 16394.
[29] G. Pages, J.F. Rouayrenc, G. Le Cam, M. Mariller, A. Le Cam, Eur. J. Biochem. 190 (1990) 385.

[30] K.X. Chai, J.-X. Ma, S.H. Murray, J. Chao, L. Chao, J. Biol. Chem. 266 (1991) 16029.
[31] K. Ohkubo, S. Ogata, Y. Misumi, N. Takami, Y. Ikehara, J. Biochem. 109 (1991) 243.
[32] R. Bouillon, H. Van Baelen, W. Rombauts, P. de Moor, J. Biol. Chem. 253 (1978) 4426.
[33] N.E. Cooke, J. Biol. Chem. 261 (1986) 3441.
[34] K. Ray, X. Wang, M. Zhao, N.E. Cooke, J. Biol. Chem. 266 (1991) 6221.
[35] S. Chao, K.X. Chai, L. Chao, J. Chao, Biochemistry 29 (1990) 323.
[36] Y. Misumi, M. Sohda, K. Ohkubo, N. Takami, K. Oda, Y.K. Ikehara, J. Biochem. 108 (1990) 230.
[37] M.S. Boguski, N.A. Elshourbagy, J.M. Taylor, J.I. Gordon, Proc. Natl. Acad. Sci. USA 81 (1984) 5021.
[38] M.S. Boguski, E.H. Birkenmeier, N.A. Elshourbagy, J.M. Taylor, J.I. Gordon, J. Biol. Chem. 261 (1986) 6398.
[39] I.A. Haddad, J.M. Ordovas, T. Fitzpatrick, S.K. Karathanas, J. Biol. Chem. 261 (1986) 13268.
[40] J.W. McLean, C. Fukazawa, J.M. Taylor, J. Biol. Chem. 258 (1983) 8993.
[41] C. Fukazawa, A. Matsumoto, L.M. Taylor, Nucleic Acids Res. 14 (1986) 9527.
[42] W.-P. Fung, G.J. Howlett, G. Schreiber, J. Biol. Chem. 261 (1986) 13777.
[43] A.H. Gordon, Biochem. J. 159 (1976) 643.
[44] B. Wärmegård, N. Martin, S. Johansson, Biochemistry 31 (1992) 2346.
[45] P.G. Harder, J.C. Jamieson, Glycobiology 7 (1997) 791.
[46] T. Xiao, D.L. DeCamp, S.R. Spran, Prot. Sci. 9 (2000) 1889.
[47] M. Rassouli, H. Sambasivam, P. Azadi, A. Dell, H.R. Morris, A. Nagpurkar, S. Mookerjea, R.K. Murray, J. Biol. Chem. 267 (1992) 2947.
[48] H. Sambasivam, M. Rassouli, R.K. Murray, A. Nagpurkar, S. Mookerjea, P. Azadi, A. Dell, H.R. Morris, J. Biol. Chem. 268 (1993) 10007.
[49] J.E. Poncin, J.A. Martial, J.E. Gielen, Eur. J. Biochem. 140 (1984) 493.
[50] B.C. Laurent, M.H.K. Nilsson, C.O. Bavik, T.A. Jones, J. Sundelin, P.A. Peterson, J. Biol. Chem. 260 (1985) 11476.
[51] J. Sundelin, B.C. Laurent, H. Anundi, L. Tragardh, D. Larhammar, L. Bjorck, U. Eriksson, B. Akerstrom, A. Jones, M. Newcomer, P.A. Peterson, L. Rask, J. Biol. Chem. 260 (1985) 6472.
[52] P.W. Dickson, G.J. Howlett, G. Schreiber, J. Biol. Chem. 260 (1985) 8214.
[53] J. Sundelin, H. Melhus, S. Das, U. Erisson, P. Lind, L. Tragardh, P.A. Peterson, K. Rask, J. Biol. Chem. 260 (1985) 6481.
[54] A. Wojtczak, Acta Biochim. Pol. 44 (1997) 505.
[55] A. Manolis, D.W. Cox, Prep. Biochem. 10 (1980) 121.
[56] R.E. Fleming, J.D. Gitlin, J. Biol. Chem. 265 (1990) 7701.
[57] J. Urban, D. Chan, G. Schreiber, J. Biol. Chem. 254 (1979) 10565.
[58] S. Furuto-Kato, A. Matsumoto, N. Kitamura, S. Nakanishi, J. Biol. Chem. 260 (1985) 12054.
[59] T. Cole, A.S. Inglis, C.M. Roxburgh, G.J. Howlett, FEBS Lett. 182 (1985) 57.
[60] W.-P. Fung, G. Schreiber, J. Biol. Chem. 262 (1987) 9298.
[61] G.A. Ricca, J.M. Taylor, J. Biol. Chem. 256 (1981) 11199.
[62] Y.C.J. Liao, J.M. Taylor, J.L. Vannice, G.A. Clawson, E.A. Smuckler, Mol. Cell. Biol. 5 (1985) 3634.
[63] M.W. Collard, M.D. Griswold, Biochemistry 26 (1987) 3297.
[64] S. Bettuzzi, R.A. Hiipakka, P. Gilna, S.T. Liao, Biochem. J. 257 (1989) 293.
[65] A. Kurosky, H.H. Kim, B. Touchstone, Comp. Biochem. Physiol. B Comp. Biochem. 55 (1976) 453.
[66] L.A. Goldstein, E.C. Heath, J. Biol. Chem. 259 (1984) 9212.
[67] J.M. Hanley, T.H. Haugen, E.C. Heath, J. Biol. Chem. 258 (1983) 7858.
[68] M. Abd-el-Fattah, R. Scherer, F.M. Fouad, G. Ruhenstroth-Bauer, Cancer Res. 41 (1981) 2548.
[69] M. Emmett, A. McCracken, J.L. Brown, A.J. Crowle, J. Immunol. Methods 67 (1984) 279.
[70] T. Kadofuku, T. Iijima, T. Sato, I. Tei, Y. Makino, J. Chromatogr. 275 (1983) 71.
[71] M. Yoshida, M. Itoh, T. Imai, Y. Tanimoto, I. Sakurabayashi, S. Furuya, Electrophoresis 12 (1991) 80.
[72] P.H. O'Farrell, J. Biol. Chem. 250 (1975) 4007.
[73] N.G. Anderson, N.L. Anderson, Anal. Biochem. 85 (1978) 331.
[74] N.L. Anderson, N.G. Anderson, Anal. Biochem. 85 (1978) 341.
[75] H. Baumann, G.L. Firestone, T.L. Burgess, K.W. Gross, K.R. Yamamoto, W.A. Held, J. Biol. Chem. 258 (1983) 563.
[76] N.L. Anderson, R. Esquer-Blanco, J.-P. Hofmann, N.G. Anderson, Electrophoresis 12 (1991) 907.
[77] N.L. Anderson, R. Esquer-Blasco, J.-P. Hofmann, L. Meheus, J. Raymackers, S. Steiner, F. Witzmann, N.G. Anderson, Electrophoresis 16 (1995) 1977.
[78] J. Szpirer, C. Szpirer, J. Cell Sci. 35 (1979) 267.
[79] P.M. Li, J.L. Zhong, R.Q. Chen, X.K. Zhang, K.L. Ho, J.F. Chiu, D.P. Huang, Int. J. Biochem. 21 (1989) 15.
[80] C.G. Duarte, G.L. dos Santos, A.E. Azzolini, A.I. de Assis Pandochi, Int. J. Immunopharmacol. 22 (2000) 25.
[81] M. Bellringer, K. Rahman, R. Coleman, Biochem. J. 249 (1988) 513.
[82] T. Marshall, O. Vesterberg, Electrophoresis 4 (1983) 363.
[83] G. Zastrow, S. Günther, W. Postel, A. Görg, H.A. Diehl, E.H.J.M. Jansen, Electrophoresis 11 (1990) 655.
[84] L. Chauvelot-Moachon, F. Delers, C. Pous, R. Engler, F. Tallet, J.P. Giroud, J. Pharmacol. Exp. Ther. 244 (1988) 1103.
[85] T. Izumi, T. Kitagawa, Chem. Pharm. Bull. 37 (1989) 742.
[86] T. Izumi, T. Nagayama, T. Kitagawa, Chem. Pharm. Bull. 37 (1989) 746.
[87] E. Okezaki, T. Terasaki, M. Nakamura, O. Nagata, H. Kato, A. Tsuji, J. Pharm. Sci. 78 (1989) 504.
[88] K. Naora, Y. Katagiri, N. Ichikawa, M. Hayashibara, K. Iwamoto, J. Pharm. Pharmacol. 42 (1990) 704.
[89] S.C. Fang, R.W. Chen, E. Fallin, Chem.-Biol. Interact. 15 (1976) 51.

[90] B.R. Martin, M.L. Reynolds, L.S. Harris, E. Toro-Goyco, Biochem. Pharmacol. 33 (1984) 429.
[91] W.C. Cole, W. Wolf, Chem.-Biol. Interact. 30 (1980) 223.
[92] C.J. Bowmer, W.E. Lindup, J. Pharmacol. Exp. Ther. 210 (1979) 440.
[93] W.G. Crouthamel, R.J. Cenedella, Pharmacology 13 (1975) 465.
[94] T. Terasaki, N. Imaeda, K. Nishide, A. Tsuji, J. Pharmacobio-Dyn. 9 (1986) 81.
[95] S. Kaul, W.A. Ritschel, Methods Find. Exp. Clin. Pharmacol. 9 (1987) 317.
[96] A.F. Aubry, N. Markoglou, A. McGann, Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 112 (1995) 257.
[97] A. Shibukawa, M. Kadohara, J.Y. He, M. Nishimura, S. Naito, T. Nakagawa, J. Chromatogr. A 694 (1995) 81.
[98] F. Herve, M.T. Martin, K. Rajkowski, P. Dessen, N. Cittanova, Biochem. J. 244 (1987) 81.
[99] F. Herve, K. Rajkowski, M.T. Martin, P. Dessen, N. Cittanova, Biochem. J. 221 (1984) 401.
[100] R.J. Jett, J.O.D. Pierce, K.L. Stemmer, Arch. Environ. Health 17 (1968) 29.
[101] P.S. Grabowski, B.K. Park, Biochem. Pharmacol. 33 (1984) 3289.
[102] J.C. Livesey, Z. Grunbaum, K.A. Krohn, Biochem. Pharmacol. 39 (1990) 1807.
[103] S. Worrall, R.G. Dickinson, Life Sci. 56 (1995) 1921.
[104] B. Marzocchi, B. Magi, L. Bini, C. Cellesi, A. Rossolini, O. Massidda, V. Pallini, Electrophoresis 16 (1995) 851.
[105] B. Magi, B. Marzocchi, L. Bini, C. Cellesi, A. Rossolini, V. Pallini, Electrophoresis 16 (1995) 1190.
[106] J.J. Harding, Adv. Prot. Chem. 37 (1985) 247.
[107] P.L. Skipper, M.W. Obiedzinski, S.R. Tannenbaum, D.W. Miller, R.K. Mitchum, F.F. Kadlubar, Cancer Res. 45 (1985) 5122.
[108] N.L. Anderson, D.C. Copple, R.A. Bendele, G.S. Probst, F.C. Richardson, Fund. Appl. Toxicol. 18 (1992) 570.
[109] A.G. Paige, K.L. Whitcomb, J. Liu, O. Kinstler, Pharmaceut. Res. 12 (1995) 1883.
[110] U. Schilling, E.A. Friedrich, H. Sinn, H.H. Schrenk, J.H. Clorius, W. Maier-Borst, Int. J. Radiat. Appl. Instr. B 19 (1992) 685.
[111] N. Dashti, J.A. Ontko, Atherosclerosis 49 (1983) 255.
[112] J. Oscarsson, S.O. Olofsson, G. Bondjers, S. Eden, Endocrinology 125 (1989) 1638.
[113] E.J. Lewis, J. Bishop, C.H. Cashin, J. Pharmacol. Methods 21 (1989) 183.
[114] A. Guglielmotti, B. Silvestrini, L. Saso, I. Zwain, C.Y. Chen, Biochem. Mol. Biol. Int. 29 (1993) 747.
[115] C.R. Self, W.E. Barber, P.J. Machin, J.M. Osbond, C.E. Smithen, B.P. Tong, J.C. Wickens, D.P. Bloxham, D. Bradshaw, C.H. Cashin et al., J. Med. Chem. 34 (1991) 772.
[116] E.J. Lewis, J. Bishop, S.J. Aspinall, Inflamm. Res. 47 (1998) 26.
[117] M. Sironi, M. Gadina, M. Kankova, F. Riganti, A. Mantovani, M. Zandalasini, P. Ghezzi, Int. J. Immunopharm. 14 (1992) 1045.
[118] V.L. Goncalves De Moraes, B.B. Vargaftig, J. Lefort, A. Maeger, M. Chignard, Br. J. Pharmacol. 117 (1996) 1792.
[119] G.D. Martich, R.L. Danner, M. Ceska, A.F. Suffredini, J. Exp. Med. 173 (1999) 1021.
[120] J.D. Sipe, L.M. Bartle, L.D. Loose, J. Immunol. 148 (1992) 480.
[121] R.N. Spengler, M.L. Spengler, P. Lincol, D.G. Remick, R.M. Strieter, S.L. Kunkel, Infect. Immun. 57 (1989) 2837.
[122] S.J. Ochalski, D.A. Hartman, M.T. Belfast, T.L. Walter, K.B. Glaser, R.P. Carlson, Agents Actions 39 (1993) C52.
[123] J. Bondeson, R. Sundler, Biochem. Pharmacol. 52 (1996) 35.
[124] S. Sacco, D. Agnello, M. Sottocorno, G. Lozza, A. Monopoli, P. Villa, P. Ghezzi, J. Neurochem. 71 (1998) 2063.
[125] A. Koj, Biochim. Biophys. Acta 1317 (1996) 84.
[126] S.P. Li, N.D. Goldman, Biochemistry 35 (1996) 9060.
[127] B. Weinhold, A. Bader, V. Poli, U. Ruther, Biochem. J. 325 (1997) 617.
[128] S.A. Berry, P.L. Bergad, A.M. Stolz, H.C. Towle, S.J. Schwarzenberg, Am. J. Physiol. 276 (1999) C1374.
[129] I. Leal-Berumen, P. O'Byrne, A. Gupta, C.D. Richards, J.D. Marshall, J. Immunol. 154 (1995) 4759.
[130] B.K. Ray, A. Ray, Biochem. Biophys. Res. Commun. 193 (1993) 1159.
[131] K. Lieb, B.L. Fiebich, H. Schaller, M. Berger, J. Bauer, J. Neurochem. 67 (1996) 2039.
[132] Y.M. Lee, L.H. Miau, C.J. Chang, S.C. Lee, Mol. Cell. Biol. 16 (1996) 4257.
[133] E. Kopp, S. Ghosh, Science 265 (1994) 956.
[134] P. Oeth, N. Mackman, Blood 86 (1995) 4144.
[135] L. Santucci, S. Fiorucci, F.M. Di Matteo, A. Morelli, Gastroenterology 108 (1995) 393.
[136] V. Bertrand, R. Guimbaud, M. Tulliez, C. Mauprivez, P. Sogni, D. Couturier, J.P. Giroud, S. Chaussade, L. Chauvelot-Moachon, Br. J. Pharmacol. 124 (1998) 1385.
[137] S.Z. Ding, S.K. Lam, S.T. Yuen, B.C. Wong, W.M. Hui, J. Ho, X. Guo, C.H. Cho, Eur. J. Pharmacol. 348 (1998) 257.
[138] G. de Jong, H.G. van Eijk, Electrophoresis 9 (1988) 589.
[139] G. de Jong, W.L. van Noort, R.A. Feelders, C.M. de Jeu-Jaspars, H.G. van Eijk, Clin. Chim. Acta 212 (1992) 27.
[140] W.A. LaFramboise, M.J. Daood, R. Guthrie, G.S. Butler-Browne, R.G. Whalen, M. Ontell, Am. J. Physiol. 259 (1990) L116.
[141] G. Evans, C.H. Wheeler, J.M. Corbett, M.J. Dunn, Electrophoresis 18 (1997) 471.
[142] F.A. Witzmann, C.D. Fultz, R.A. Grant, L.S. Wright, S.E. Kornguth, F.L. Siegel, Electrophoresis 19 (1998) 2491.
[143] P. Tietz, P.C. de Groen, N.L. Anderson, C. Sims, R. Esquer-Blasco, L. Meheus, J. Raymackers, M. Dauwe, N.F. LaRusso, Electrophoresis 19 (1998) 3207.
[144] M.J. Hubbard, Electrophoresis 19 (1998) 1891.
[145] X. Li, K.P. Pleissner, C. Scheler, V. Regitz-Zagrosek, J. Salnikow, P.R. Jungblut, Electrophoresis 20 (1999) 891.
[146] D.B. Kristensen, M. Inamatsu, T. Matsuzaki, K. Yoshizato, Exp. Dermatol. 8 (1999) 339.

[147] M. Fountoulakis, E. Schuller, R. Hardmeier, P. Berndt, G. Lubec, Electrophoresis 20 (1999) 3572.
[148] M. Fountoulakis, R. Hardmeier, R.H. Hoge, G. Lubec, Exp. Neurol. 167 (2001) 86.
[149] D.B. Kristensen, N. Kawada, K. Imamura, Y. Miyamoto, C. Tateno, S. Seki, T. Kuroki, K. Yoshizato, Hepatology 32 (2000) 268.
[150] R.J. Isfort, R.T. Hinkle, M.B. Jones, F. Wang, K.D. Greis, Y. Sun, T.W. Keough, N.L. Anderson, R.J. Sheldon, Electrophoresis 21 (2000) 2228.
[151] V. Felipo, M.D. Minana, I. Azorin, S. Grisolia, J. Neurochem. 51 (1988) 1041.
[152] P.J. Wirth, O. Vesterberg, Electrophoresis 9 (1988) 47.
[153] W.G. Hinson, J.L. Pipkin, J.F. Anson, D.L. Davies, E.R. Burns, D.A. Casciano, Appl. Theor. Electrophoresis 2 (1991) 93.
[154] B.S. Eckert, P.L. Yeagle, Cell Motil. Cytoskel. 33 (1996) 30.
[155] Y. Egashira, A. Tanabe, T. Ohta, H. Sanada, J. Nutr. Sci. Vitaminol. 44 (1998) 129.
[156] E.J. Schoenle, L.D. Adams, D.W. Sammons, J. Biol. Chem. 259 (1984) 12112.
[157] L.A. Pon, N.R. Orme-Johnson, J. Biol. Chem. 261 (1986) 6594.
[158] M.J. McCroskey, P.A. Pierce-Ramsey, J.R. Colca, J.D. Pearson, Biochim. Biophys. Acta 1011 (1989) 212.
[159] M. Raymondjean, B. Kneip, J. Kruh, Biochim. Biophys. Acta 741 (1983) 30.
[160] R. Shanker, W.E. Neeley, W.H. Dillmann, J. Mol. Cell Cardiol. 19 (1987) 595.
[161] J. Knopp, J. Brtko, P. Filipcik, Endocr. Regulat. 26 (1992) 67.
[162] Y. Wang, A. Xu, G.J. Cooper, Biochem. J. 344 (1999) 971.
[163] B. Antoine, A. Rahimi-Pour, G. Siest, J. Magdalou, M.M. Galteau, Cell Biochem. Function 5 (1987) 217.
[164] T.J. Huang, G.M. Trakshel, M.D. Maines, Arch. Biochem. Biophys. 274 (1989) 617.
[165] T.J. Huang, M.D. Maines, Mol. Pharmacol. 37 (1990) 25.
[166] X. Guitart, E.J. Nestler, Brain Res. 516 (1990) 57.
[167] D.G. Myszka, R.P. Swenson, J. Biol. Chem. 266 (1991) 20725.
[168] F.C. Richardson, S.C. Strom, D.M. Copple, R.A. Bendele, G.S. Probst, N.L. Anderson, Electrophoresis 14 (1993) 157.
[169] F.C. Richardson, D.M. Hom, N.L. Anderson, Carcinogenesis 15 (1994) 325.
[170] F.A. Witzmann, D.N. Parker, B.M. Jarnot, Toxicol. Lett. 71 (1994) 271.
[171] S. Steiner, D. Wahl, B.L. Mangold, R. Robison, J. Raymackers, L. Meheus, N.L. Anderson, A. Cordier, Biochem. Biophys. Res. Commun. 218 (1996) 777.
[172] L. Aicher, D. Wahl, A. Arce, O. Grenet, S. Steiner, Electrophoresis 19 (1998) 1998.
[173] F.A. Witzmann, C.D. Fultz, R.A. Grant, L.S. Wright, S.E. Kornguth, F.L. Siegel, Electrophoresis 20 (1999) 943.
[174] P. Cutler, D.J. Bell, H.C. Birrell, J.C. Connelly, S.C. Connor, E. Holmes, B.C. Mitchell, S.Y. Monte, B.A. Neville, R. Pickford, S. Polley, K. Schneider, J.M. Skehel, Electrophoresis 20 (1999) 3647.
[175] S. Chevalier, N. Macdonald, R. Tonge, S. Rayner, R. Rowlinson, J. Shaw, J. Young, M. Davison, R.A. Roberts, Eur. J. Biochem. 267 (2000) 4624.
[176] S. Steiner, C.L. Gatlin, J.J. Lennon, A.M. McGrath, A.M. Aponte, A.J. Makusky, M.C. Rohrs, N.L. Anderson, Electrophoresis 21 (2000) 2129.
[177] S. Steiner, C.L. Gatlin, J.J. Lennon, A.M. McGrath, M.D. Seonarain, A.J. Makusky, A.M. Aponte, R. Esquer-Blasco, N.L. Anderson, Toxicol. Lett. 120 (2001) 369.
[178] T.H. Rushmore, L. Harris, M. Nagai, R.N. Sharma, M.A. Hayes, R.G. Cameron, R.K. Murray, E. Farber, Cancer Res. 48 (1988) 2805.
[179] E. Zeindl-Eberhart, H.M. Rabes, Carcinogenesis 13 (1992) 1177.
[180] E. Zeindl-Eberhart, P. Jungblut, H.M. Rabes, Electrophoresis 15 (1994) 372.
[181] E. Zeindl-Eberhart, P.R. Jungblut, A. Otto, H.M. Rabes, J. Biol. Chem. 269 (1994) 14589.
[182] E. Zeindl-Eberhart, P.R. Jungblut, A. Otto, R. Kerler, H.M. Rabes, Eur. J. Biochem. 247 (1997) 792.
[183] F.L. Rodkey, Clin. Chem. 11 (1965) 478.
[184] B.T. Doumas, W.A. Watson, H.G. Biggs, Clin. Chim. Acta 31 (1971) 87.
[185] E. Gianazza, P. De Ponti, Electrophoresis 14 (1993) 1259.
[186] B. Herbert, M. Galvani, M. Hamdan, E. Olivieri, J. MacCarthy, S. Pedersen, P.G. Righetti, Electrophoresis 22 (2001) 2046.
[187] M. Galvani, M. Hamdan, B. Herbert, P.G. Righetti, Electrophoresis 22 (2001) 2058.
[188] M. Galvani, L. Rovatti, M. Hamdan, B. Herbert, P.G. Righetti, Electrophoresis 22 (2001) 2066.
[189] E. Gianazza, S. Astrua-Testori, P. Giacon, P.G. Righetti, Electrophoresis 6 (1985) 332.
[190] A.J. Link, 2-D Proteome Analysis Protocols, Humana Press, Totowa, 1998.
[191] U.K. Laemmli, Nature 227 (1970) 680.
[192] A. Görg, W. Postel, J. Weser, S. Günther, S.R. Strahler, S.M. Hanash, L. Somerlot, Electrophoresis 8 (1987) 122.
[193] J.I. Garrels, J. Biol. Chem. 25 (1989) 5269.