Comp. Biochem. Physiol. Vol. 95B, No. 4, pp. 803–810, 1990
Printed in Great Britain

0305-0491/90 $3.00 + 0.00

SERUM PROTEINS OF RHINOCEROSES: INTER- AND INTRA-SPECIFIC VARIATION

A. STRATIL,* P. BOBÁK,* P. KALÁB,* D. ČÍŽOVÁ* and R. POKORNÝ†

*Czechoslovak Academy of Sciences, Institute of Animal Physiology and Genetics, 277 21 Liběchov, Czechoslovakia; and †East Bohemian Zoological Garden, 544 01 Dvůr Králové, Czechoslovakia

(*Received* 29 *August* 1989)

Abstract—1. Serum proteins of *Ceratotherium simum cottoni* Lydekker (*C. s. cottoni*), *Diceros bicornis* L. (*D. bicornis*) and *Rhinoceros unicornis* L. (*R. unicornis*) were studied by 1D PAGE, 2D agarose–PAGE, immunoblotting and inhibitions of trypsin and chymotrypsin.

2. In all species studied albumin, transferrin, α_1B glycoprotein, vitamin D binding protein (GC), α_2HS glycoprotein, haptoglobin, haemopexin, ceruloplasmin, esterase and protease inhibitors were found.

3. 1D PAGE and 2D agarose–PAGE patterns of serum proteins of rhinoceroses were found to be species-specific.

4. In *C. s. cottoni* intra-specific variation was observed in vitamin D binding protein (GC), protease inhibitors AC and ATC2 and haptoglobin. Less well defined variation was also detected in protease inhibitor ATC1, a postalbumin (PSA) and an esterase (ES3).

INTRODUCTION

At present there are five surviving species of rhinoceroses belonging to four genera—two Asian, *Rhinoceros* and *Dicerorhinus*, and two African, *Diceros* and *Ceratotherium* (Dobroruka *et al.*, 1975). Living rhinoceroses are the last evolutionary links of relatively invariable lines of *Perissodactyla*. Their phylogeny can be traced back to Eocene–Oligocene (Špinar and Burian, 1984). According to fossil evidence African and Asian genera split about 26 Ma; *Diceros* and *Ceratotherium* diverged from their congeneric ancestor about 8 Ma (Merenlender *et al.*, 1989).

Until now little attention has been devoted to studies of rhinoceros proteins, and to inter- and intra-specific differences. This is easily understood, as the number of rhinoceroses in the wild are low, and difficult to obtain. Even in zoological parks these animals are rare. For example, *C. s. cottoni* survive as a single population, with only 17–20 animals in Garamba National Park in Zaire (Ryder *et al.*, not dated), and only about a dozen animals maintained in captivity (du Toit *et al.*, 1987).

Osterhoff and Keep (1970), using starch gel electrophoresis, detected differences between southern white rhinoceros and black rhinoceros in their haemoglobin, transferrin and albumin. In southern white rhinoceros they also described intra-specific variation in transferrin, prealbumin and esterase. Merenlender *et al.* (1989) studied 25–31 allozymic loci in tissue and blood samples in African and Asian rhinoceroses and used the data to calculate genetic distances between the various taxa. Polymorphism was observed only in aspartate aminotransferase (*C. s. simum*), unidentified plasma protein, AB-3 (*D. bicornis*, *C. s. simum* and *C. s. cottoni*), and phosphoglucomutase-2 (*D. bicornis*, *C. s. simum* and *C. s. cottoni*).

The aim of this study was the identification and interspecies comparison of serum proteins in three species of rhinoceros, *C. s. cottoni*, *D. bicornis* and *R. unicornis*, and a search for possible genetic variation in *C. s. cottoni*. Species-specific patterns were observed in all three studied taxa. In *C. s. cottoni* intra-specific variation, suggesting genetic polymorphism, was observed in protease inhibitors AC and ATC2, vitamin D binding protein (GC) and haptoglobin; less clear was the variation in protease inhibitor ATC1, a postalbumin (PSA) and an esterase (ES3).

MATERIALS AND METHODS

Animals

Northern white rhinoceros, *C. s. cottoni* (six animals), black rhinoceros, *D. bicornis* (one animal) and Indian rhinoceros, *R. unicornis*, 1758 (one animal) maintained at the Zoological Park in Dvůr Králové (Czechoslovakia) were studied. Blood samples were collected from *vena auricularis*, after immobilization of the animals with 1–1.2 ml of LA Immobilon (Reckit and Coleman, UK). Serum samples were kept frozen at −20°C. For comparison horse (*Equus caballus*) serum was used.

1D and 2D gel electrophoresis

One-dimensional polyacrylamide gel electrophoresis (1D PAGE) and two-dimensional agarose (pH 5.4)–polyacrylamide gel (pH 9.0) electrophoresis (2D agarose–PAGE) were performed as described by Juneja and Gahne (1987). In 1D PAGE serum samples were applied after soaking into single pieces of filter paper (Whatman No. 3). The gels were stained with Coomassie Blue G-250. Haemopexin and haptoglobin were detected with the benzidine staining after prior addition of alkaline haematin (Stratil *et al.*, 1984) and chicken haemoglobin to the serum samples, respectively. Esterase was stained by using 100 ml of 0.08 M Tris–citrate buffer, pH 6.5, containing 100 mg of α-naphtylacetate and 100 mg of Fast Blue BB salt.

Detection of inhibitors of trypsin and chymotrypsin

A method of Pellegrini *et al.* (1984), adapted by Gahne and Juneja (1985), was used, details were obtained from Gahne *et al.* (personal communication).

Polyacrylamide gels with 12, 4 and 8% layers of acrylamide were prepared according to Juneja and Gahne (1987), with the exception that to the 12% layer 36.5 mg of fibrinogen was added [Type I–S from bovine plasma, (Sigma) or bovine fibrinogen, (Imuna, Šarišské Michalány, Czechoslovakia)]. After 2D agarose–PAGE was completed the gel was incubated for 2 hr at laboratory temperature in 200 ml of buffer solution, 0.2 M triethanolamine–HCl, 20 mM $CaCl_2$, pH 7.8, containing 4 mg of α-chymotrypsin (69 U/mg), or 2.6 mg trypsin from bovine pancreas (40 U/mg). After incubation the solution was discarded and the gel was rinsed overnight in an excess of distilled water (using a shaker), and then stained with Coomassie Blue G-250.

Starch gel electrophoresis

Horizontal starch gel electrophoresis with water cooling was used. Albumins were separated in a discontinuous Tris–citrate, pH 6.9, and boric acid–NaOH, pH 8.6, buffer system (Tucker, 1968). Autoradiography of transferrin using ^{59}Fe citrate was performed after separation of serum samples in a Tris–citrate–boric acid–LiOH buffer system of Valenta *et al.* (1976).

Immunoblotting

Serum proteins after 1D PAGE or 2D agarose–PAGE were transferred to nitrocellulose sheets (Synpor 6, Barvylaky, Praha) by 45 min passive blotting, simultaneously from upper and lower surfaces of the gel (Stratil *et al.*, 1988). α_2HS glycoprotein, haptoglobin and ceruloplasmin were detected after 75 min semi-dry electrophoretic transfer (using LKB Novablot, Electrophoretic Transfer Kit), in 25 mM Tris, 192 mM glycine, 20% methanol (Towbin *et al.*, 1979). The blots were blocked with 2% Tween-20 in phosphate buffered saline (2% TPBS) by washing for 2 min (Bjerrum *et al.*, 1987) and then incubated with different primary antisera diluted in 0.05% TPBS, which could be supplemented with 2.5% polyethyleneglycol 6000 (PEG) to enhance the sensitivity of some weaker primary antibody reactions. Incubation was with one of the following secondary peroxidase-labelled (Px) antisera; swine anti-rabbit immunoglobulin (SwAR/Px) or rabbit anti-swine immuno-globulin (RASw/Px) (both Sevac, Praha), diluted in 0.05% TPBS. Diaminobenzidine tetrahydrochloride/H_2O_2/$CoCl_2$ solution (Nibbering and van Furth, 1987) was used as a substrate for the peroxidase reaction.

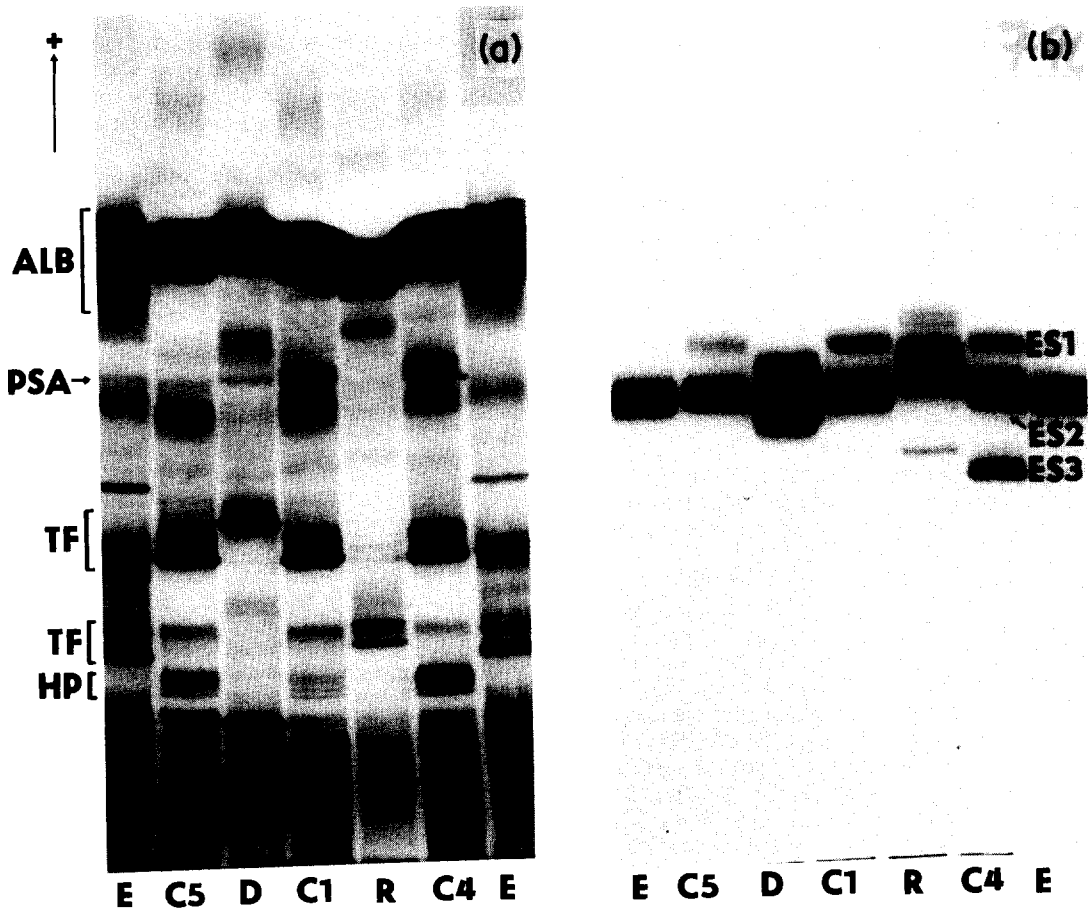

Fig. 1. 1D PAGE of serum proteins of *E. caballus* (E), *C. s. cottoni* (C), *D. bicornis* (D) and *R. unicornis* (R). (For numbers C1, C4 and C5 see Table 1.) (A) Coomassie Blue staining. ALB = albumin; TF = transferrin; HP = haptoglobin; PSA = postalbumin of *C. s. cottoni*. (B) Esterase staining. Note an extra esterase zone (ES3) in C4.

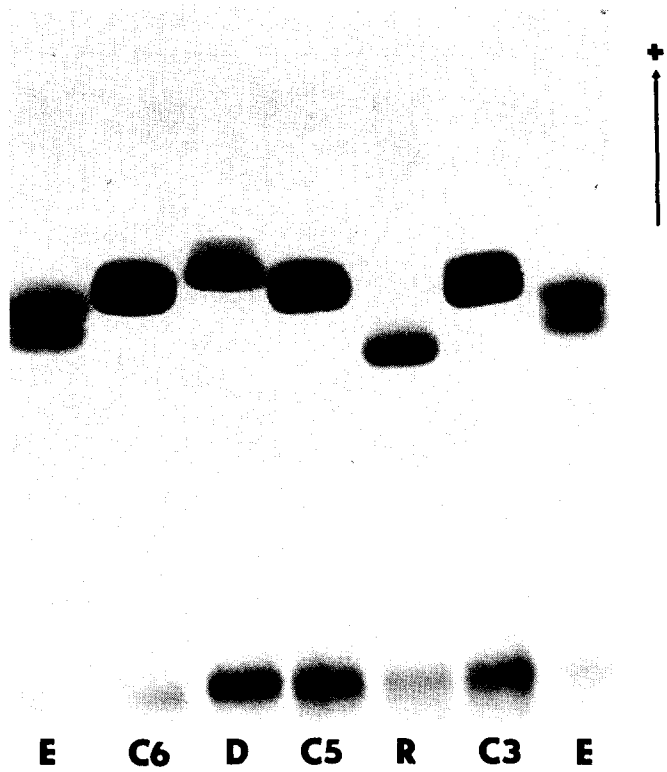

Fig. 2. Starch gel electrophoresis of horse and rhinoceroses serum samples to show albumin patterns. Designation of species is as in Fig. 1. Horse albumin is of FS phenotype.

The following antisera were used: rabbit anti-pig α_2HS glycoprotein (dilution 1:250; 2.5% PEG; SwAR/Px 1:500); rabbit anti-pig α_1B-glycoprotein (1:500; SwAR/Px 1:500); rabbit anti-human GC (1:250; SwAR/Px 1:500); swine anti-human haptoglobin (1:100; 2.5% PEG; RASw/Px 1:500); swine anti-human ceruloplasmin (1:200; 2.5% PEG; RASw/Px 1:500); rabbit anti-sheep transferrin (1 min passive transfer; 1:2000; SwAR/Px 1:500).

Antisera to pig α_2HS, α_1B and human GC were prepared by immunizing rabbits with respective protein zones cut out from 2D agarose–PAGE, after separation of crude serum fractions (Stratil *et al.*, 1988). Antiserum to sheep transferrin was obtained after immunizing rabbits with isolated sheep transferrin. Antisera to haptoglobin and ceruloplasmin were obtained from Sevac (Praha).

RESULTS

1D *PAGE—inter-species comparison*

Figure 1A shows a comparison of protein patterns of *C. s. cottoni*, *D. bicornis*, *R. unicornis* and the horse. The patterns were species-specific, with almost all visible zones of the species differing. The patterns of *C. s. cottoni* and *D. bicornis* were more similar to each other than to that of *R. unicornis*. The two African species were characterized by strong zones of transferrin, which were localized approximately in the middle, between the boundary of the 12% gel and albumin. In *R. unicornis* transferrin had a slower migration and had two zones of similar intensity.

In the region between albumin and transferrin many protein zones, which mutually overlapped, were located (see below). By using specific staining, esterase could be shown (Fig. 1B). 1D PAGE could also successfully be used for immunoblotting with the use of specific antisera (see below).

Although differences in albumins of various species could be seen in 1D PAGE, a much clearer separation was obtained by using starch gel electrophoresis (Fig. 2).

2D *agarose–PAGE—inter-species comparison and identification of proteins*

Serum protein patterns after 2D agarose–PAGE of *C. s. cottoni*, *D. bicornis*, *R. unicornis* and the horse are shown in Fig. 3A. Horse serum proteins were designated according to Juneja *et al.* (1984), except for protease inhibitors. For identification of proteins in rhinoceroses the following methods were used: immunoblotting [vitamin D binding protein (GC), α_1B, α_2HS, transferrin (TF), ceruloplasmin (CP), haemopexin (HPX) and haptoglobin (HP)], autoradiography with ^{59}Fe (transferrin in starch gel electrophoresis), inhibition activities to trypsin and chymotrypsin, (protease inhibitors AT1, AT2, AT3, AC, ATC1 and ATC2), specific staining for esterase [esterase (ES)] and benzidine staining (haemopexin and haptoglobin).

In all studied species albumin (ALB), TF, α_1*B*, vitamin D binding protein (GC), α_2HS, HP, HPX, CP, ES, and protease inhibitors AT, AC, ATC were found. As can be seen in Figs 3B and C, the patterns of protease inhibitors are very complex and homologies cannot be unequivocally determined just on the basis of inhibitory spectra. Therefore, the designation of Pi1 and Pi2 of Juneja *et al.* (1984), as used for the

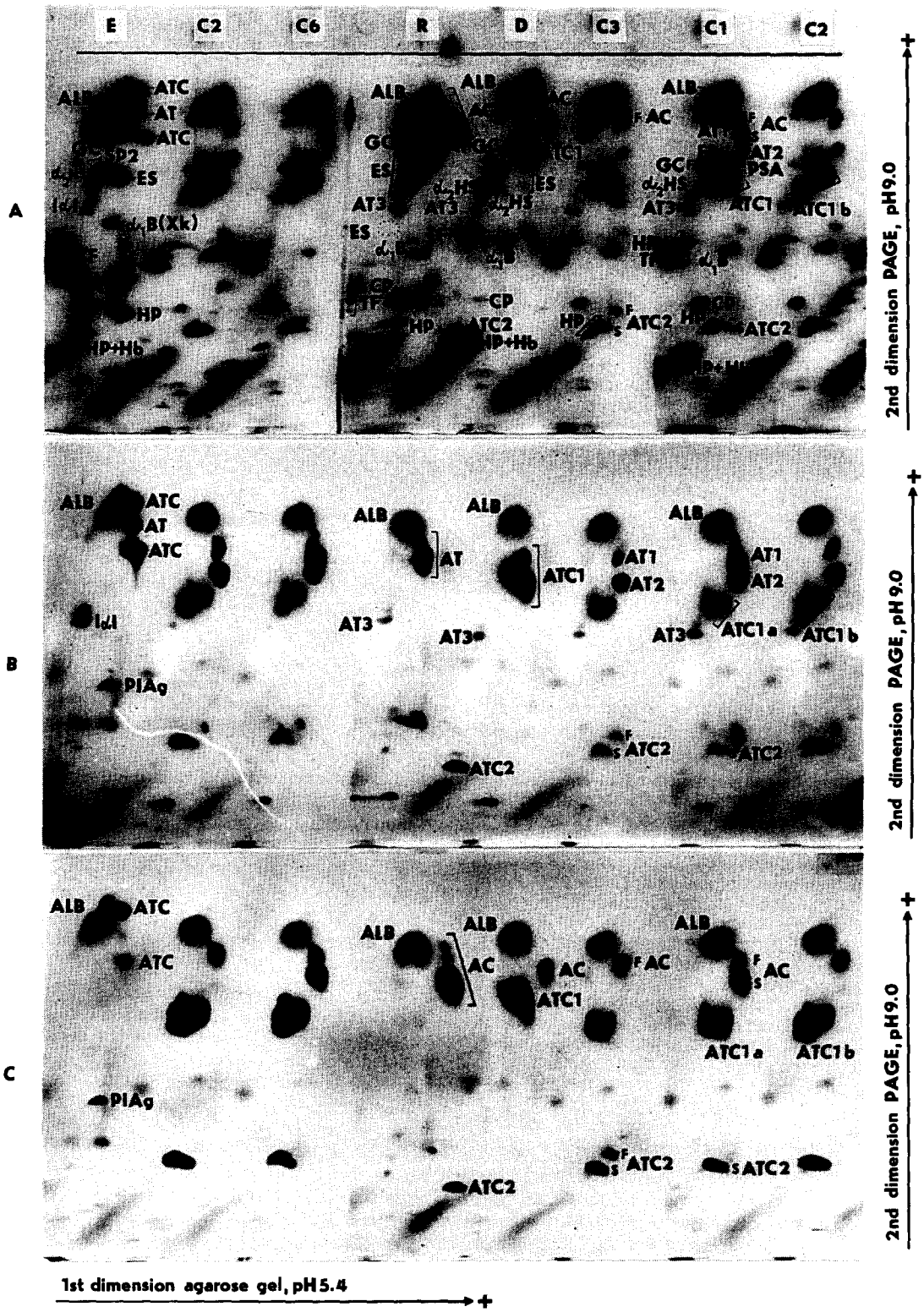

Fig. 3. 2D agarose–PAGE of serum proteins of horse and rhinoceroses. Designation of species is as in Fig. 1, and designations C2, C3, C4 and C6 correspond to *C. s. cottoni* numbers given in Table 1. ALB = albumin; GC = vitamin D binding protein; α_2HS = α_2HS glycoprotein; α_1B = α_1B glycoprotein; HPX = haemopexin; TF = transferrin; CP = ceruloplasmin; HP = free haptoglobin; HP + Hb = haptoglobin–haemoglobin complex; AT = inhibitors of trypsin; AC = inhibitors of chymotrypsin; ATC = inhibitors of trypsin and chymotrypsin; ES = esterase; PSA = postalbumin; SP2 = horse apolipoprotein A-IV (Juneja *et al.*, 1989); IαI = horse inter-α-trypsin inhibitor; PIAg = horse protease inhibitor aggregate. The same serum samples were separated in all three gels. (A) Coomassie Blue staining, (B) inhibition of trypsin, (C) inhibition of chymotrypsin.

horse, is not used here; we use symbols AT, AC and ATC, which designate inhibition of trypsin (AT), chymotrypsin (AC) or both trypsin and chymotrypsin (ATC). Only for AT3 do we assume that it is homologous to horse inter-α-trypsin inhibitor, (IαI) (Pollitt and Bell, 1983). In *R. unicornis* a slowly migrating protease inhibitor ATC2 was not observed in either 1D PAGE or 2D agarose–PAGE.

In addition to the identified proteins there were, in sera of all three rhinoceros species, further zones of unidentified proteins. Of special interest was a postalbumin, PSA (see Fig. 3A) which was present in some, but not all, *C. s. cottoni*, but was not clearly seen in either the other species of rhinoceroses or the horse.

Intra-specific variation in C. s. cottoni

In *C. s. cottoni*, in spite of studying only six animals, differences indicating genetic polymorphism were observed in GC, protease inhibitors AC and ATC2 and HP. Variation was detected also in protease inhibitor ATC1, a postalbumin (PSA) and an esterase (ES3).

In the GC system two phenotypes were observed, F and FS. In Fig. 3A only phenotype F is shown. Both phenotypes, as well as inter-species differences, are shown in immunoblotting following 1D PAGE in Fig. 4. GC appears to be a codominant system.

The two observed phenotypes of protease inhibitor AC, F and FS, are clearly seen in Figs 3A and C. The phenotypes of ATC2, S and FS, are best seen in Fig. 3C. Variant ATC2 S, as well as variant AC S, had a higher inhibitory activity than F variants. When Coomassie Blue staining was used (Fig. 3A) ATC2 S variant was located just to the right from free HP, and in some cases it overlapped with HP. It was clearly seen when chicken haemoglobin was added to serum samples and zones of free HP disappeared.

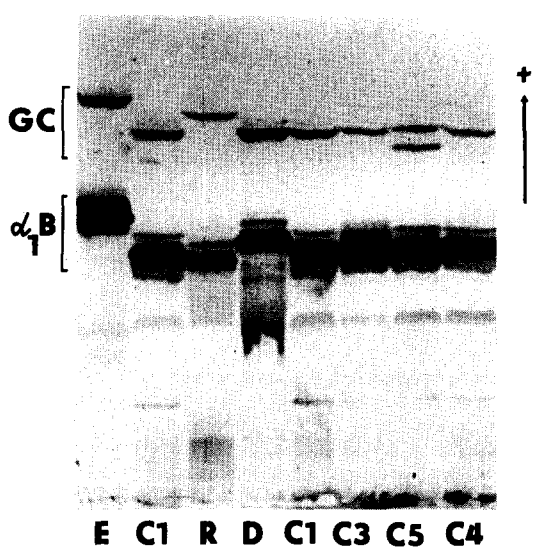

Fig. 4. Immunoblotting of horse and rhinoceroses sera following 1D PAGE. A mixture of rabbit antisera to human GC and pig α_1B was used as primary antibody in a dilution given in Materials and Methods. Designation of species is as in Fig. 1. Note inter-specific variation in both proteins and polymorphism of GC of *C. s. cottoni* (sample C5 is heterozygote FS, others are homozygotes F).

Both protease inhibitors, AC as well as ATC2, appear to be codominant systems.

In free HP four phenotypes were observed, F, I, FI and IS. The HP phenotypes are best seen in 1D PAGE (Figs 5A and B). Phenotype F had three zones almost fusing together, the middle one being very strong. Phenotype I was slower and had three closely spaced zones, phenotype FI was as a mixture of F and I, and phenotype IS had five to six closely spaced zones. Although it is tempting to consider F, I and S as variants, this system will require a study on more animals as well as genetic evidence.

There was also some further variation in serum proteins of *C. s. cottoni*, which was difficult to interpret. Four animals had a strong zone of PSA and two had not (see Figs 1A, 3A and 5A). PSA was clearly seen both in 1D PAGE and 2D agarose–PAGE. In ATC1 some animals had the two spots more closely spaced (type a), and in others these spots were further apart (type b) (Figs 3A–C). There could also be overlapping with some other zones in Coomassie Blue stained gels. Differences occurred also in prealbumin patterns. After esterase staining one animal had an extra zone (ES3) that was slower than the main band (see Fig. 1B), but in this case we were not sure if it was genetic polymorphism.

The intra-specific differences observed in the serum protein patterns of six *C. s. cottoni* are summarized in Table 1.

DISCUSSION

Comparisons of 1D PAGE and 2D agarose–PAGE patterns of all three rhinoceros species showed that each species had a specific pattern, and positions of almost all homologous proteins differed. Of all proteins studied there seemed to be a similar mobility of GC and protease inhibitors AC and AT3 in *C. s. cottoni* and *D. bicornis*. In *D. bicornis* and *R. unicornis*, as well as in the horse, an unidentified protein, PSA, was not seen. Protease inhibitors of all three species were rather complex and it was difficult to identify homologies between different rhinoceros species and the horse. It seems that AT3 could be a homologue of horse inter-α-trypsin inhibitor, IαI (Pollitt and Bell, 1983; Juneja *et al.*, 1984). *R. unicornis* lacks protease inhibitor ATC2.

Intra-specific variation of serum proteins observed in *C. s. cottoni* is surprisingly high in view of the low number of animals tested, and in view of previously published data. Osterhoff and Keep (1970) observed in *C. s. simum* variations in transferrin, prealbumins and esterase. However, in prealbumins and esterase they did not get a clear differentiation. In transferrin they observed two variants in *C. s. simum* and one variant in *D. bicornis*. The transferrin of the latter had the same mobility as the fast variant of *C. s. simum*. The authors did not present sufficient experimental details of starch gel electrophoretic technique used and it is not clear whether they attempted to identify transferrin. According to our results transferrin of *D. bicornis* has a faster anodic migration than that of *C. s. cottoni* in conventional alkaline buffers, both in starch gel and polyacrylamide gel electrophoresis. Therefore, transferrin could correspond to their slower invariant zone (see Fig. 2 of Osterhoff and Keep, 1970).

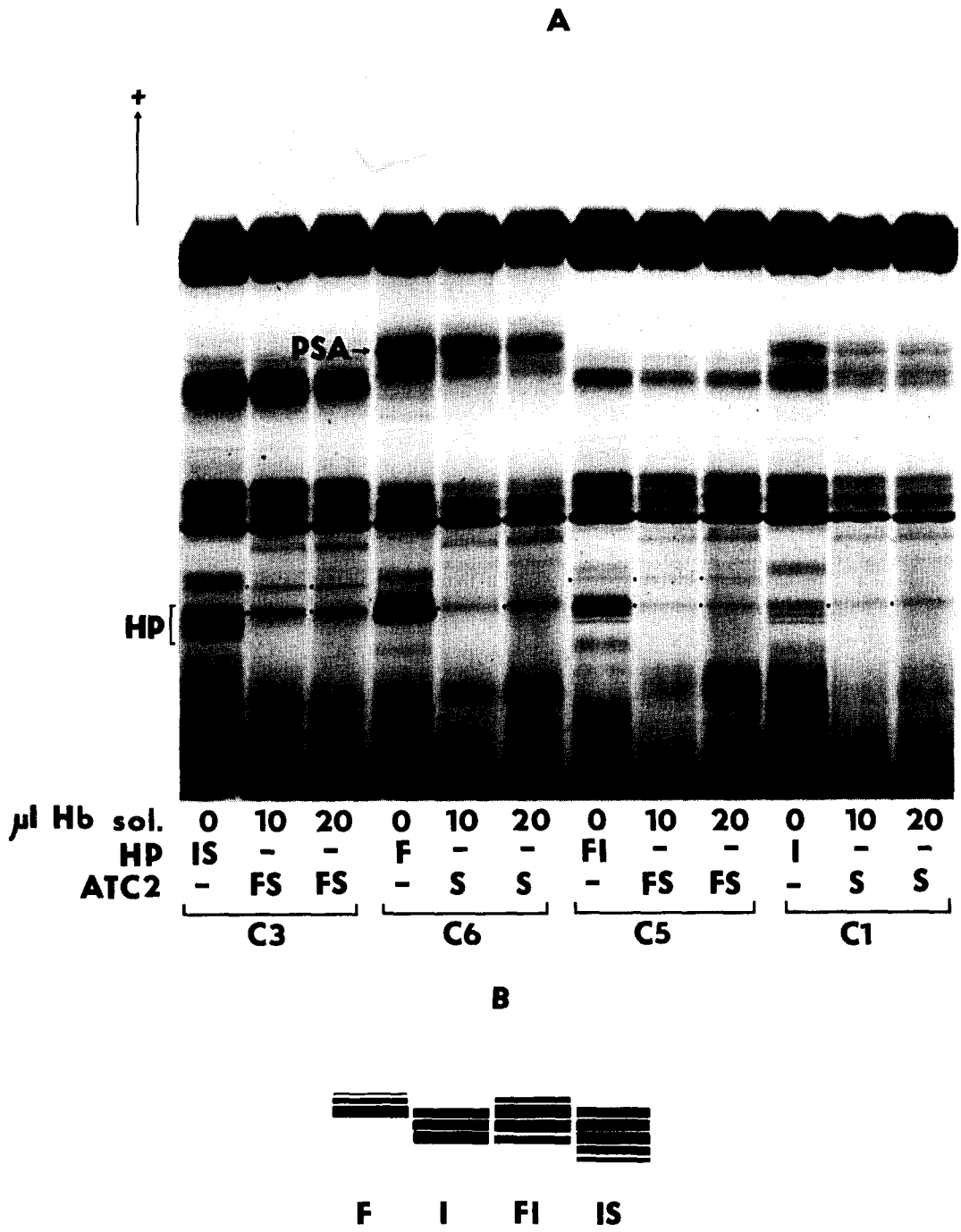

Fig. 5. (A) 1D PAGE of serum proteins of *C. s. cottoni* showing polymorphism of haptoglobin (HP), and protease inhibitor ATC2. Zones of ATC2 are marked with dots. Note that free haptoglobin overlaps with variant ATC2 S. ATC2 phenotypes are clearly seen in serum samples to which haemoglobin had been added. Haptoglobin phenotypes are seen only in samples without added haemoglobin. There was no difference when 10 or 20 µl of haemoglobin solution had been added. Note also the differences in PSA zones between individual animals. (B) Schematic diagram of four observed haptoglobin phenotypes of *C. s. cottoni*.

Merenlender *et al.* (1989) observed polymorphism of only a single polymorphic protein (AB-3) in serum of all three taxa of African rhinoceroses. They suspected that this protein could be transferrin. Although they did not present any experimental details we are convinced that their AB-3 could not be transferrin, this is because we studied the same animals of *C. s. cottoni* that originated from The Zoological Park in Dvůr Králové, and we did not observe any sign of polymorphism in the transferrin.

The 1D PAGE and 2D agarose–PAGE used in this study are useful methods for detection of polymor-

Table 1. Intra-specific variability in seven serum proteins of *C. s. cottoni* maintained in Dvůr Králové Zoo

No.	Name	Phenotypes of AC	GC	ATC2	HP	PSA	ATC1	ES3
1	Súdán	FS	F	S	I	+	a	–
2	Nádí	F	F	S	IS	+	b	–
3	Nésári	F	F	FS	IS	–	a	–
4	Nasi	FS	F	S	FI	+	a	+
5	Suni	F	FS	FS	FI	–	a	–
6	Ben	FS	F	S	F	+	b	–

phic proteins in rhinoceroses. With these methods polymorphism of protease inhibitors AC, ATC2, GC and HP can easily be studied. For some proteins 1D PAGE is better. Haptoglobin polymorphism is clearly seen in samples without haemoglobin, and ATC2 polymorphism can be reliably typed after addition of haemoglobin to serum samples, as HP–Hb complex has a slower migration and does not overlap with ATC2 S variant. Variation of PSA is also better seen in 1D PAGE. It is also possible that PSA could be a single codominant system, of which we can only see the fast variant while the slow variant is overlapped with protease inhibitor ATC1 and other proteins, so that it is impossible to read the phenotypes.

Merenlender *et al.* (1989), who tested 31 enzyme and protein systems in blood and tissues of four taxa of rhinoceroses used the data for calculating genetic distances between them. They observed extremely small amounts of intra-specific variation. As we analyzed three species, and also some of the same animals of northern white rhinoceroses as Merenlender *et al.*, we can conclude that there are pronounced differences between different species, at least in serum proteins, and the intra-specific variation in *C. s. cottoni* is much greater than formerly described. Without any doubt, the use of 1D PAGE, 2D agarose–PAGE and immunoblotting (and possibly isoelectric focusing), adds increased sophistication and sensitivity to the analysis of genetic variation in rhinoceroses. Using these techniques could provide a greater chance of correlating fossil evidence and the results obtained by studying proteins and mitochondrial DNA (George *et al.*, 1983; du Toit *et al.*, 1987; Merenlender *et al.*, 1989).

It is recommended that similar approaches as those presented, or recommended, in this paper are used for comparison of *C. s. cottoni* and *C. s. simum* blood proteins, to find whether or not the two taxa differ. Although the two subspecies can display differences that can be in fact normal intra-specific polymorphisms, inter-specific differences could exist that would distinguish the two subspecies. Such cases are not unusual in other closely related species. For example, there is a clear-cut difference in isoelectric focusing of haemoglobin Bs of mouflon and sheep, whilst other blood proteins of these two species do not differ in starch gel electrophoresis (Stratil and Bobák, 1988; Tucker and Clarke, 1980).

Acknowledgements—We thank Dr J. Váhala for collecting blood samples of all rhinoceroses studied, and Dr O. A. Ryder for critical reading of the manuscript.

REFERENCES

Bjerrum O. J., Selmer J. C. and Lihme A. (1987) Native immunoblotting: transfer of membrane proteins in the presence of non-ionic detergent. *Electrophoresis* **8,** 388–397.

Dobroruka L. J., Fejfar O., Gaisler J., Hanák V., Hanzák J., Heráň I., Mazák V., Veselovský Z. and Volf J. (1975) Přehled soustavy a české názvy savců. (An outline classification of mammals and their Czech names.) *Lynx (Praha)*, Suppl. IV.

du Toit R. F., Foose T. J. and Cumming D. H. M. (Editors) (1987) *Proceedings of African Rhino Workshop, Cincinnatti.* Pachyderm No. 9.

Gahne B. and Juneja R. K. (1985) Close genetic linkage between four plasma α-protease inhibitor loci (*Pi1, Po1A, Po1B, Pi2*) in pigs. *Protides Biol. Fluids* **33,** 119–122.

George M., Puentes L. A. and Ryder O. A. (1983) Genetische Unterschiede zwischen den Unserarten des Breitmaulnashorns. *International Studbook of African Rhinoceroses*, Vol. 2, pp. 60–67. Zoological Garten, Berlin.

Juneja R. K., Andersson L., Sandberg K., Gahne B., Adelsteinsson S. and Gunnarsson E. (1984) Two-dimensional electrophoresis of horse serum proteins: genetic polymorphism of ceruloplasmin and two other serum proteins. *Anim. Blood Grps Biochem. Genet.* **15,** 237–250.

Juneja R. K. and Gahne B. (1987) Simultaneous phenotyping of pig plasma α-protease inhibitors (PI1, PO1A, PO1B, PI2) and four other proteins (PO2, TF, CP, HPX) by a simple method of 2-D horizontal electrophoresis. *Anim. Genet.* **18,** 197–211.

Juneja R. K., Gahne B., Lukka M. and Ehnholm C. (1989) A previously reported polymorphic plasma protein of dogs and horses, identified as apolipoprotein A-IV. *Anim. Genet.* **20,** 59–63.

Merenlender A. M., Woodruff D. S., Ryder O. A., Kock R. and Váhala J. (1989) Allozyme variation and differentiation in African and Indian rhinoceroses. *J. Hered.* **80,** 377–382.

Niberring P. H. and van Furth R. (1987) Microphotometric quantitation of the reaction product of several indirect immunoperoxidase methods demonstrating monoclonal antibody binding to antigens immobilized on nitrocellulose. *J. Histochem. Cytochem.* **35,** 1425–1431.

Osterhoff D. R. and Keep M. E. (1970) Natural variation in the blood proteins of white and black rhinos. *Lammergeyer* **11,** 50–53.

Pellegrini A., Hägeli G., Fretz D. and von Fellenberg R. (1984) Natural protease inhibitors: qualitative and quantitative assay by fibrinogen–agarose electrophoresis. *Analyt. Biochem.* **138,** 335–339.

Pollitt C. C. and Bell K. (1983) Characterization of the α_1-protease inhibitor system in thoroughbred horse plasma by horizontal two-dimensional (ISO-DALT) electrophoresis. 1. Protein staining. *Anim. Blood Grps Biochem. Genet.* **14,** 83–105.

Stratil A. and Bobák P. (1988) Comparison of biochemical polymorphisms in mouflon and sheep: isoelectric differences in haemoglobins and quantitative variation of mouflon haemopexin. *Comp. Biochem. Physiol.* **90B,** 159–162.

Stratil A., Glasnák V., Tomášek V., Williams J. and Clamp J. R. (1984) Haemopexin in sheep, mouflon and goat: genetic polymorphism, heterogeneity and partial characterization. *Anim. Blood Grps Biochem. Genet.* **15,** 285–297.

Stratil A., Kaláb P. and Pokorný R. (1988) Evidence for the presence of α_1B-glycoprotein in mammalian sera: immunoblotting studies. *Comp. Biochem. Physiol.* **91B,** 783–788.

Špinar Z. V. and Burian Z. (1984) *Paleontologie Obratlovců. (The Paleontology of Vertebrates.)* (In Czech). Academia, Praha.

Towbin H., Staehlin T. and Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. natn. Acad. Sci. USA* **76,** 4450–4454.

Tucker E. M. (1968) Serum albumin polymorphism in sheep. *Vox Sang. (Basel)* **15,** 306–308.

Tucker E. M. and Clarke S. W. (1980) Comparative aspects of biochemical polymorphism in the blood of Caprinae species and their hybrids. *Anim. Blood Grps Biochem. Genet.* **11,** 163–183.

Valenta M., Stratil A., Šlechtová V., Kálal L. and Šlechta V. (1976) Polymorphism of transferrin in carp (*Cyprinus carpio* L.): Genetic determination, isolation and partial characterization. *Biochem. Genet.* **14,** 27–45.