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Comparative Protein Analysis of Non-salmonid Fish Rhabdoviruses

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SUMMARY

On the basis of gel precipitation, reactions with rabbit anti-pike fry rhabdovirus sera, a recent virus isolate (V75/94) from pike fry, and the grass carp rhabdovirus were found to be indistinguishable from pike fry rhabdovirus. The proteins of these viruses were compared to those of spring viraemia of carp virus. In 'rocket' immunoelectrophoresis the pike fry rhabdoviruses and the grass carp rhabdovirus showed up to 4 lines with homologous antiserum in which the precipitates formed by the G and M proteins were fused. With this antiserum, spring viraemia of carp virus gave only one precipitation arc which was formed by the N protein. All the viruses are very similar to vesicular stomatitis virus with respect to the mol. wt., localization and phosphorylation of structural proteins. In polyacrylamide–SDS slab gels the viruses showed minor differences in the migration of their respective NS proteins.

INTRODUCTION

Vertebrate rhabdoviruses have been subdivided into two genera, Vesiculovirus and Lyssavirus (Fenner, 1976). This classification is supported by observations on the virion polypeptides (see, e.g., Sokol & Koprowski, 1975). Using this criterion, rhabdoviruses of fish have also been classified in the two genera: the salmonid viruses causing infectious hematopoietic necrosis (IHN) and viral haemorrhagic septicaemia (VHS) appear rabies-like, whereas spring viraemia of carp (SVC; Fijan *et al.* 1971) and pike fry rhabdovirus (PFR; de Kinkelin *et al.* 1973) appear similar to vesicular stomatitis virus (Hill *et al.* 1975; Lenoir & de Kinkelin, 1975; McAllister & Wagner, 1975; Roy *et al.* 1975; Sokol & Koprowski, 1975). For the two salmonid rhabdoviruses it has been shown that they can be distinguished on the basis of the mol. wt. of their structural proteins (McAllister & Wagner, 1975).

The aim of the present paper was to study the protein composition of non-salmonid rhabdoviruses in a comparative manner and to examine the antigenic relatedness of their proteins. The viruses examined comprise PFR, a recent field isolate of this virus designated V75/94, SVC and the recently described rhabdovirus from grass carp (GRV; Ahne, 1975). Since the comparison of these viruses by means of conventional neutralization tests was hampered by a number of unexpected phenomena, these results will be published separately (Clerx *et al.* 1978).

METHODS

Viruses. SVC and PFR were kindly supplied by Dr P. de Kinkelin, Thiverval-Grignon, France. GRV was a gift from Dr W. Ahne, Munich, West Germany. The PFR-like virus designated V75/94 was isolated from pike fry (*Esox lucius* L.) of 5 to 6 cm total length,

symptoms of the haemorrhagic form of pike fry rhabdovirus disease (Bootsma, 1971; Bootsma *et al.* 1975). The fish were provided by courtesy of Dr R. Bootsma, Utrecht. All subsequent experiments were performed using plaque purified virus. Viruses were concentrated and purified as described earlier (Clerx *et al.* 1975). Protein was determined by the Lowry method using bovine serum albumin as a standard (Lowry *et al.* 1951).

Preparation of antisera. Two random bred rabbits were immunized by repeated intramuscular and subcutaneous injections at 4 to 8 week intervals using 0·05 to 0·4 mg of gradient purified PFR over a 1 year period. One rabbit received infectious virus, the other formalin-fixed virus (1 % formalin by volume). The first injections were given with Freund's complete adjuvant; all later immunizations were made with Freund's incomplete adjuvant (Difco). Final bleeding was performed two weeks after the last injections.

Virus labelling. To discriminate between membrane and non-membrane proteins, intact or 0·1 % Triton X-100 (Serva, Heidelberg, W. Germany) disrupted virus was iodinated by the lactoperoxidase technique at room temperature (Moore *et al.* 1974). Iodination was stopped by adding SDS and heating to 100 °C for 1 min. Chloramine T labelling was also performed as described for vesicular stomatitis virus (Moore *et al.* 1974). Free iodine was removed by extensive dialysis against phosphate buffered saline, pH 7·2. Total protein labelling was performed in cells incubated under MEM containing 2 % foetal bovine serum and 20 % of the normal amount of methionine. One h p.i. 20 μCi/ml of ^{35}S-methionine (more than 200 Ci/mmol) were added to the cultures. Labelling of viruses with ^{32}P was performed as described earlier (Clerx *et al.* 1975). All isotopes were purchased from the Radiochemical Centre, Amersham, U.K.

Electrophoresis of virus proteins in gels containing antiserum. Concentrated purified viruses in TES buffer (0·1 M-NaCl, 1 mM-EDTA, 0·02 M-tris, pH 7·4) were disrupted by adding sodium deoxycholate (Merck, Darmstadt, G.F.R.), Triton X-100 and dithiothreitol (Sigma, St Louis, U.S.A.) to give final concentrations of 2 %, 2 % and 10 mM respectively. The mixtures were incubated for 2 h at 37 °C before application to the gels. 'Fused rocket' immunoelectrophoresis was performed in 1 % agarose gels in Svendsen buffer (Weeke, 1973) containing 0·1 % Berol (Emu Werke, Copenhagen, Denmark). After diffusion of virus proteins into agarose without antiserum, an antiserum-containing gel was cast and the proteins electrophoresed for 16 to 20 h at 1·25 V/cm. Gels were further processed as described by Weeke (1973). Precipitation lines were correlated to virus polypeptides by excision of stained precipitates of ^{35}S-methionine labelled proteins, overnight elution in 2 % SDS and subsequent identification on polyacrylamide slab gels using labelled PFR as a reference. Labelled polypeptides were visualized after autoradiography of dried gels.

SDS-polyacrylamide gel electrophoresis (PAGE). Mol. wt. determinations of virus proteins were performed in slab gels with polyacrylamide concentrations ranging from 7 to 11 %, prepared as described by Laemmli (1970). Viruses were disrupted by boiling for 2 min in 2 % SDS, 5 % mercaptoethanol, 0·05 M-tris-HCl, pH 6·8. Markers for the calculation of the mol. wt. involved mono-, di-, tri- and tetrameric bovine serum albumin; in addition, *E. coli* RNA-polymerase subunits, phosphorylase A, glutamate dehydrogenase, ovalbumin, aldolase, alfalfa mosaic virus coat protein, trypsin inhibitor from soy bean and cytochrome *c* (Boehringer, Mannheim, W. Germany) were employed. Bromophenol blue served as a tracking dye. Polypeptides were separated at 100 V for 3 h (Zeegers *et al.* 1976). Virus proteins were classified according to Wagner *et al.* (1972).

Table 1. *Apparent molecular weights* ($\times 10^{-3}$) *of virion polypeptides**

Protein	Virus			
	PFR	V75/94	GRV	SVC
L	180	180	180	180
G	77–78†	76–77	76–77	76–77
NS	58	56	57	52–53
N	46–47	46–47	46–47	46–47
M	23–26	23–26	23–26	25–27

* As determined in the Laemmli gel system (1970) at 11 % concentration of acrylamide with the electrophoresis equipment described by Studier (1973) using marker proteins of mol. wt. 268, 201, 165, 155, 134, 94, 67, 53, 43, 40, 24·5, 21·5, 16 and 11×10^3.

† In 7 % polyacrylamide gels only slightly higher values were found (79 to 80×10^3).

RESULTS

Protein composition

All viruses used in this study were shown by SDS-PAGE to contain five structural proteins typical of the genus Vesiculovirus (Fenner, 1976): the large L protein, the glycosylated envelope protein G, a phosphorylated NS protein, the nucleocapsid protein N and the membrane protein M. Table 1 summarizes the mol. wt. values determined for the virion polypeptides by SDS–PAGE. In the Laemmli (1970) gel system the NS proteins of all viruses showed distinct differences in migration; they were always well separated from the N protein (Fig. 1). Considerable amounts of ^{32}P were incorporated *in vivo* into the NS proteins of all the viruses, whereas radioactive phosphate in other proteins could only be detected by autoradiography or prolonged liquid scintillation counting. As can be seen in Fig. 2, PFR, GRV and SVC are largely devoid of detectable amounts of ^{32}P co-migrating with the N protein.

Protein localization

Enzymic iodination of intact virus particles resulted in predominant labelling of the G proteins of PFR (Fig. 3), V75/94, GRV and SVC. This confirms their position at the outside of the virus membrane (Roy *et al.* 1975). They are also the only proteins affected by density gradient centrifugation: the amount of G protein diminishes by this treatment as judged by a decrease in iodine binding capacity (up to 46 % in PFR) and staining by Coomassie blue (not quantified).

When non-ionic detergents like Triton X-100, Triton N-101 or Tween 80 were incorporated into the iodination reaction mixture, there was an increase in the relative amount of label bound to the M, NS and N proteins, in this order (Fig. 3*c*). Chloramine T labelling resulted in labelling of all virus proteins (Fig. 3*b*), although the labelling of the L protein could only be demonstrated by autoradiography.

Electrophoresis of virus proteins in gels containing anti-PFR serum

Rocket immunoelectrophoresis of virus proteins was hampered by the fact that only two proteins showed mobilities which allowed them to enter the part of the gel containing antiserum. Disrupted PFR electrophoresed into agarose gels containing anti-PFR serum revealed four precipitation lines which were fairly well separated from one another. Three of them could be identified as the major proteins G, M and N as described in the Methods section (data not shown). From the gels, the highest of the two rockets (Fig. 4, arrow)

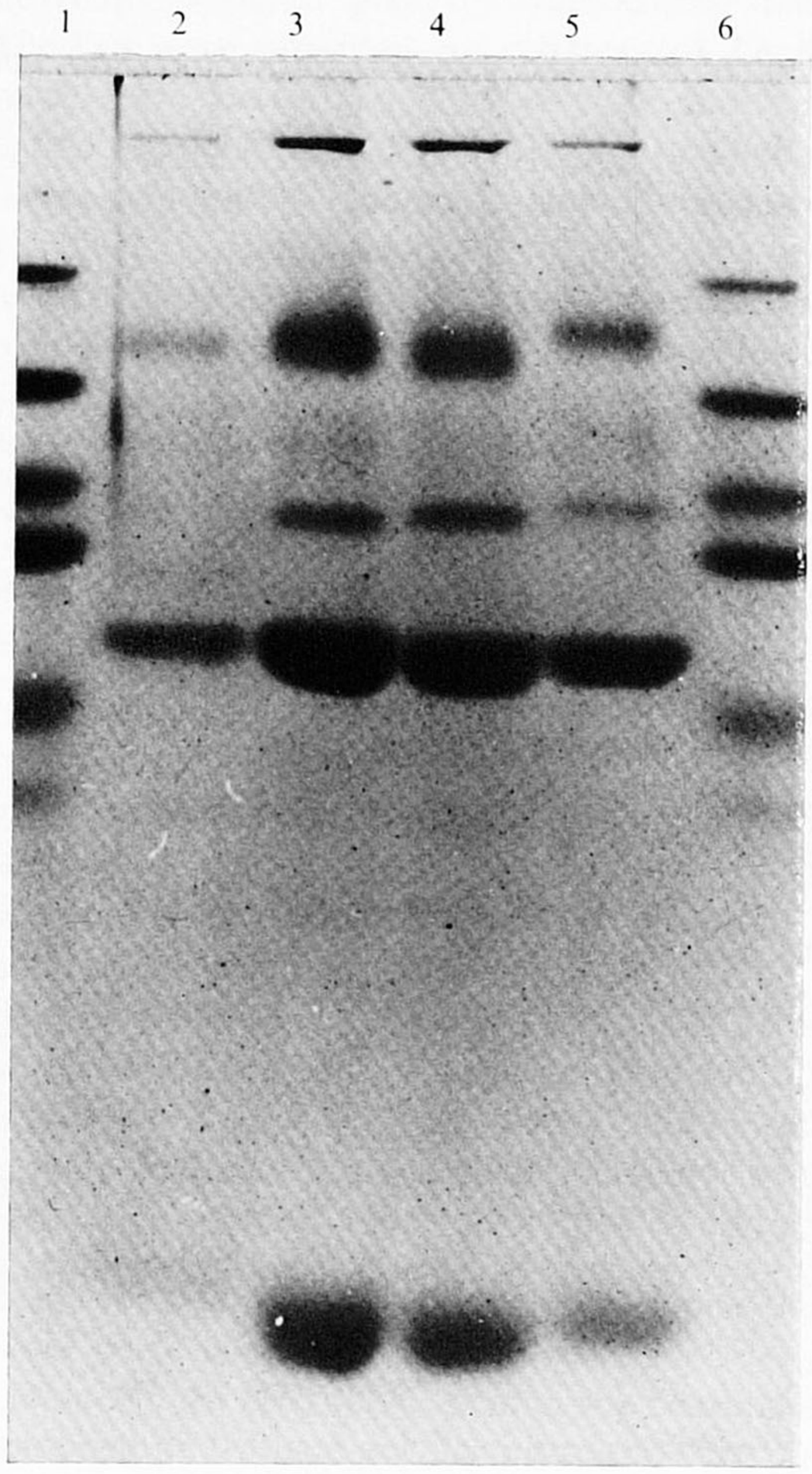

Fig. 1. Coomassie blue stained slab gel (11 % acrylamide, 0·2 % bisacrylamide) showing the migration of the proteins of SVC (lane 2), V75/94 (lane 3), GRV (lane 4), PFR (lane 5) and marker proteins of 94, 67, 60, 53, 43 and 40×10^3 (lane 1 and 6).

appeared to be composed of the G protein and rabbit heavy and light immunoglobulin chains, whereas the lower one contained the M protein. The heavy precipitate near the application well was formed by both N and NS proteins in which the NS was located at the front edge as demonstrated with ^{32}P-labelled virus. Just above this precipitate, a faint line was discernible which is a candidate for the L protein. However, the amount of label recovered after excision was too small to allow identification on slab gels. In the absence of antiserum all proteins showed a much faster migration; therefore, the low mobilities of the N and NS proteins cannot be attributed to interaction with the virus RNA. As with PFR, four precipitation lines were found using V75/94 and GRV as antigens. A reaction of identity between the G and M proteins from PFR, V75/94 and GRV could be observed since these proteins formed fused rockets (Fig. 4). The grass carp virus showed one difference with respect to PFR and V75/94: the thin precipitation line above the N/NS arc was sharper and better resolved. The same quantity of SVC proteins electrophoresed into the gel revealed only one precipitation line which could be identified as the N protein.

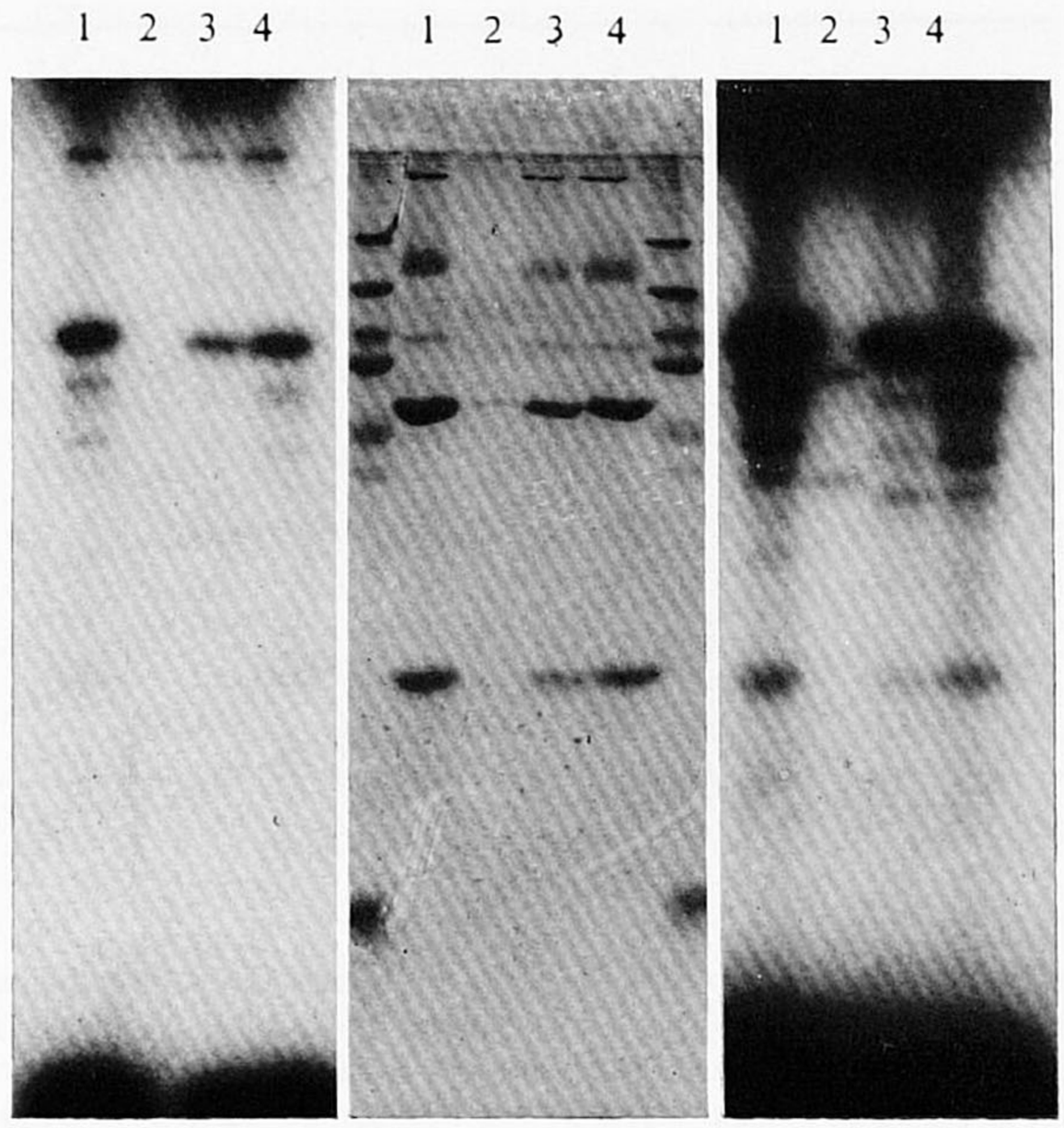

Fig. 2. Coomassie brilliant blue stained gel (centre) and two corresponding autoradiographs showing ^{32}P-labelled proteins of PFR (lane 1), SVC (lane 2), GRV (lane 3) and V75/94 (lane 4). Kodak Blue Brand X-ray film was exposed for 20 h (left) and $7\frac{1}{2}$ days to reveal SVC phosphoproteins (right).

DISCUSSION

On the basis of serum neutralization tests PFR was identified as a rhabdovirus distinct from SVC (de Kinkelin *et al.* 1973, 1974; Hill *et al.* 1975; Roy *et al.* 1975). However, it was the lack of neutralization of PFR by anti-SVC sera which provided evidence of unrelatedness rather than positive neutralizing activity of anti-PFR sera. Ahne (1975) reported the isolation of a rhabdovirus from a cyprinid host which also was not neutralized by anti-SVC serum. Since our rabbit anti-PFR sera did not show neutralizing activity, we compared the proteins of PFR, V75/94, GRV and SVC using electrophoretic and gel precipitation techniques with the aim of classifying the grass carp isolate.

The data reported here support the distinct position of SVC. It could be distinguished from the other viruses by a faster migration of its NS protein and a slower migration of the M protein in SDS-polyacrylamide gel electrophoresis. Surprisingly, the NS protein of V75/94, another isolate from pike fry exhibited a migration different from that of the reference strain of PFR (Fig. 1). This difference could be shown reproducibly with virus from different passage levels. The NS protein of GRV migrated to a position intermediate between those of PFR and V75/94. In contrast, five rhabdovirus specimens isolated from carp with swim bladder inflamation (which were a gift from Dr W. Ahne, Munich, W. Germany) showed migrations of their NS proteins identical with the reference strain of SVC (data not shown).

Since the migration of rhabdovirus NS proteins in SDS-PAGE depends on the gel system (Moore *et al.* 1974; Sokol *et al.* 1974; Both *et al.* 1975; Roy *et al.* 1975; Sokol & Clarke, 1975) the mol. wt. values of this protein should be regarded with caution. In fact, gel filtration in 6 M-guanidinium-hydrochloride indicated a mol. wt. lower than that of the N protein (our unpublished observations). Similar small differences were observed with the G proteins: PFR G protein migrated more slowly than those of V75/94, GRV and SVC.

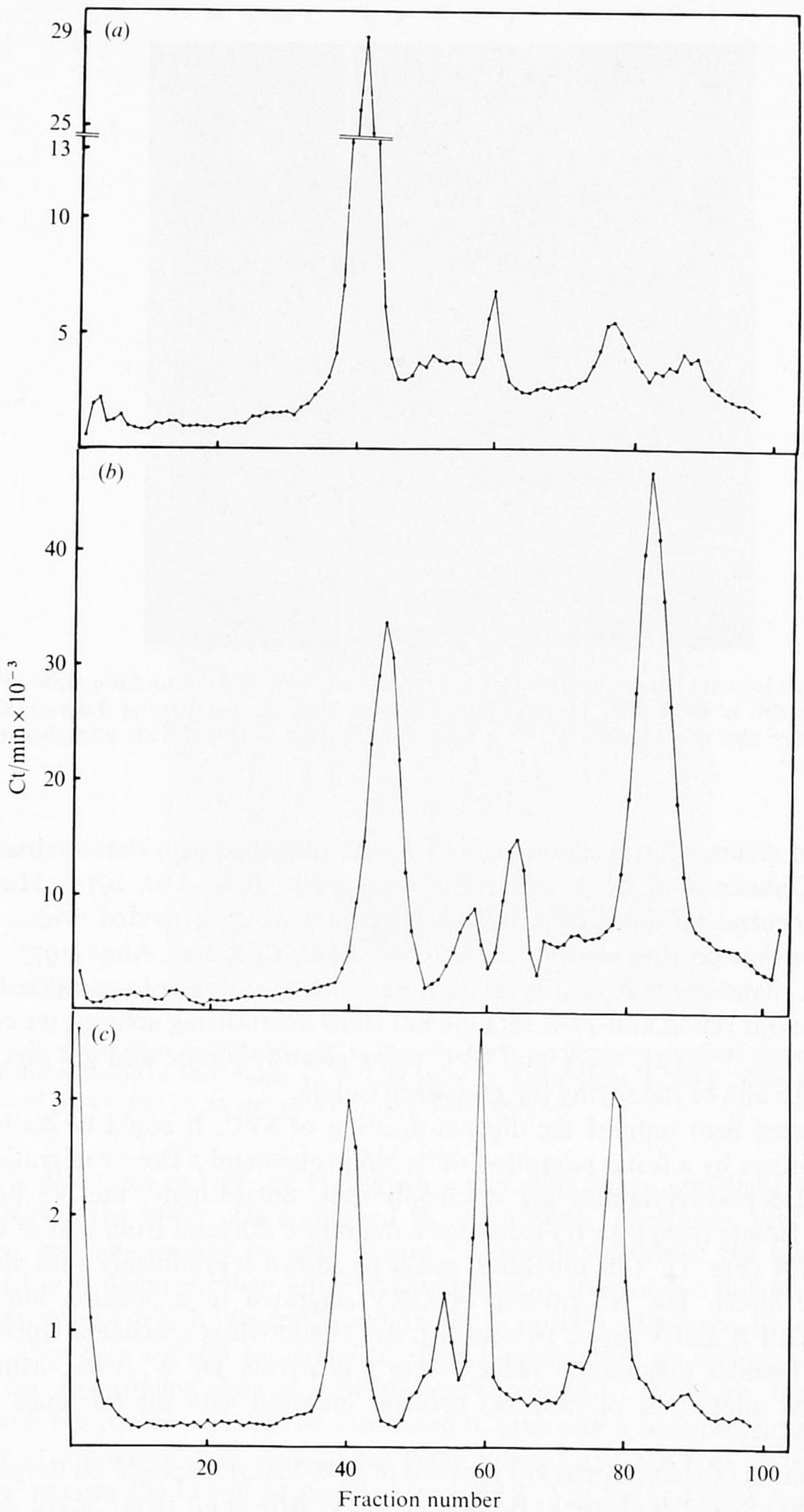

Fig. 3. Iodination of PFR. Freshly purified virus was iodinated for 20 min at room temperature by means of lactoperoxidase in the absence (*a*) and presence (*b*) of 0·1 % Triton X-100, or (*c*) with chloramine T in the presence of 0·1 % Triton X-100. Discontinuous 7 % cylindrical gels (Zeegers *et al.* 1976) were fractionated into 1 mm slices and counted in a Triton–toluene based scintillation cocktail.

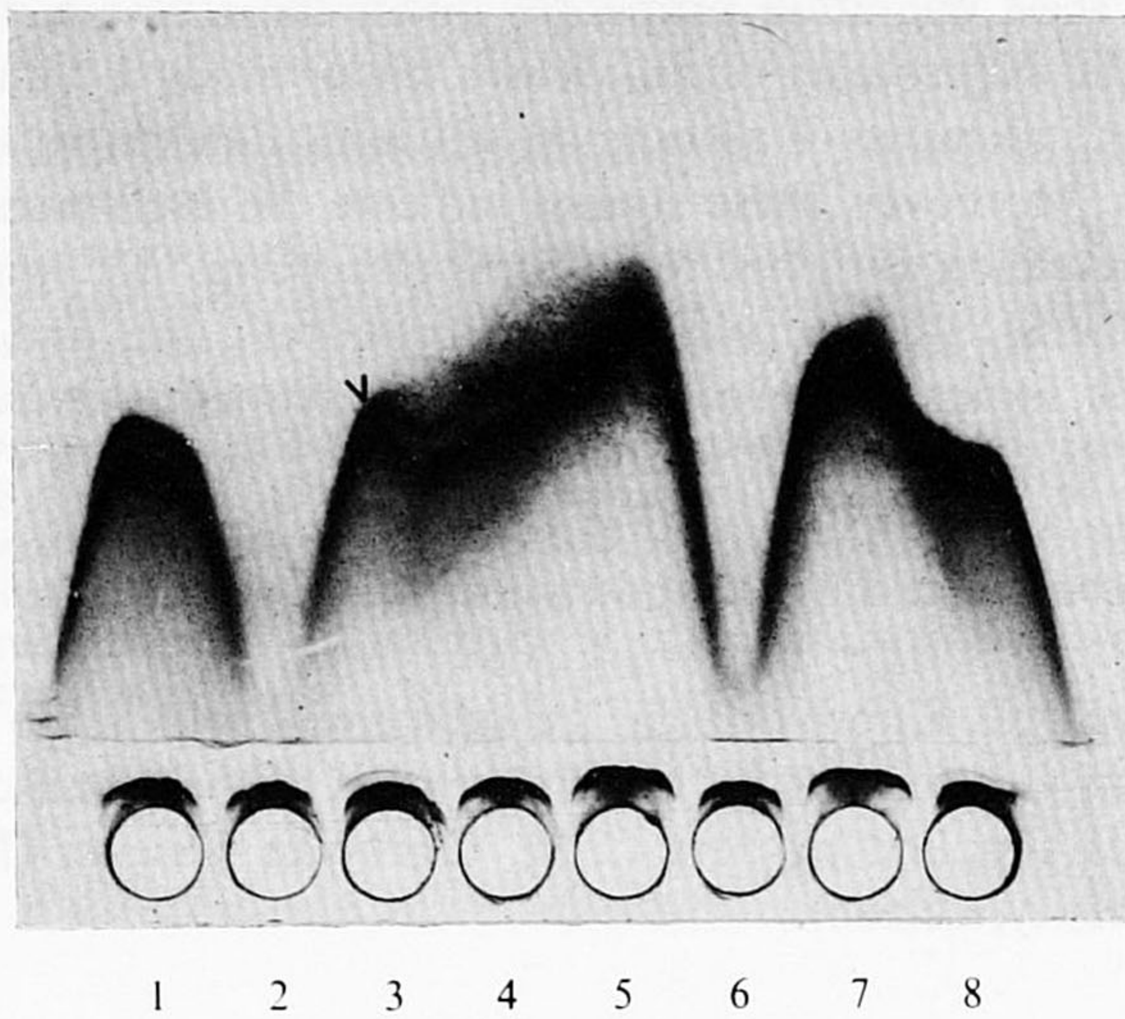

Fig. 4. Fused rocket immunoelectrophoresis of PFR-like viruses and SVC. Disrupted viruses (0·04 to 0·06 mg protein for the PFR-like viruses and 0·065 mg for SVC) were applied to the wells (3 mm diam.) 60 min before electrophoresis into the antiserum containing part of the gel (at 1·25 V/cm for 18 h). The lower part of the gel contained 0·1 % Berol and 1 % agarose in barbital-glycin/tris buffer, pH 8·8 (ionic strength 0·02). The upper part contained in addition 7 % anti-PFR rabbit serum. Antigenic relatedness is demonstrated by fusion of the precipitation lines formed by the G proteins (arrow) and M proteins of PFR (wells 1 and 4), V75/94 (wells 5 and 7) and GRV (wells 3 and 8). Wells 2 and 6: SVC.

The localization of the virion proteins of all the viruses is essentially the same as that documented for vesicular stomatitis virus (Moore *et al.* 1974; Wagner *et al.* 1975). Whereas the glycoprotein is readily labelled in intact virus preparations, the M protein becomes accessible to enzymic iodination only after disruption of the envelope. The PFR iodination data shown in Fig. 3 support a model in which the rhabdovirus NS protein is located at the surface of the nucleocapsid (Wagner *et al.* 1975) since it can be labelled almost completely after disruption of the membrane whereas the N protein under these conditions remains largely inaccessible to enzymic iodination (compare Fig. 3*b* and *c*). The pattern of *in vivo* phosphorylation of PFR, GRV, V75/94 and SVC closely parallels that of vesicular stomatitis virus in which only the NS is a phosphoprotein (Sokol *et al.* 1974; Roy *et al.* 1975; Sokol & Clarke, 1975). Phosphorylation of other structural proteins varied somewhat between different preparations, the M protein always being second in degree of phosphorylation. In contrast to other reports (Sokol *et al.* 1974; Sokol & Clarke, 1975; Sokol & Koprowski, 1975) the N protein is not the predominantly phosphorylated protein in SVC. The second ^{32}P-containing protein in SVC shows a mol. wt. of 38 to 39 × 10^3 (Fig. 2). In many similar experiments we were unable to detect any ^{32}P-containing protein co-migrating with the N protein of SVC.

In all the viruses studied, varying numbers of phosphorylated proteins become apparent after longer exposure of X-ray film which are not visible after staining (Fig. 2). Since all viruses were grown under identical conditions the differences in migration between the viruses suggest a viral rather than a cellular origin.

The immunoelectrophoresis studies clearly indicate the relatedness of PFR, V75/94 and GRV and the separate position of SVC in which only the N protein is precipitated by rabbit anti-PFR serum. A reaction of antigenic identity was noted for the G and M proteins of the non-carp viruses. Antigenic relatedness of the other proteins could not be

measured because of their migration properties under these circumstances. Nevertheless, the precipitation of the N proteins of all viruses under study by an antiserum directed against one of them is indicative of a common antigenic determinant(s) preserved on the nucleocapsid proteins. Moreover, these studies indicate the usefulness of immunoelectrophoresis methods in revealing antigenic relatedness of proteins not comparable by neutralization assays. Both the SDS-polyacrylamide electrophoretic data and those from 'rocket' immunoelectrophoresis indicate a high degree of relatedness between PFR and GRV. These results, as well as heterologous neutralization data using GRV and pike anti-PFR immune sera (Clerx *et al.* 1978) suggest that GRV must be regarded as a strain of PFR rather than a third non-salmonid fish rhabdovirus.

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