A SIMPLE PROCEDURE FOR THE ANALYSIS OF THE STRUCTURAL PROTEINS OF INFLUENZA AND PARAINFLUENZA VIRUSES INVOLVING ADSORPTION TO ERYTHROCYTES

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A simple procedure for the analysis of the structural proteins of influenza and parainfluenza viruses utilizing adsorption to erythrocytes is described. The method involves virus growth in the presence of [^{35}S]methionine, adsorption of clarified culture medium with a 0.5% suspension of either guinea-pig or chicken erythrocytes and analysis of the virus–erythrocyte aggregates by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). All of the structural proteins can be detected using this procedure, and the protein profiles of virus-adsorbed erythrocyte complexes compare extremely well with those of sucrose density gradient purified virus preparations.

influenza virus   parainfluenza virus   erythrocyte adsorption   structural proteins

INTRODUCTION

The phenomenon of virus haemagglutination was first recognized in the early 1940's with the discovery that allantoic fluid harvested from chick embryos infected with influenza virus would agglutinate chicken erythrocytes (Hirst, 1941; McClelland and Hare, 1941). Subsequently it was found that haemagglutination was a characteristic of a wide range of vertebrate viruses, including the orthomyxoviruses, paramyxoviruses, togaviruses, rhabdoviruses, coronaviruses, bunyaviruses, picornaviruses, papovaviruses, adenoviruses, parvoviruses and reoviruses. Haemagglutinating viruses adsorb to the surface receptors on the surface of a variety of avian and vertebrate erythrocyte species (for details see Andrewes et al., 1978). Chicken, guinea-pig and human erythrocytes appear to be more widely susceptible to agglutination than cells from other species. The haemagglutination (HA) and haemagglutination inhibition (HI) tests are now widely used as rapid quantitative assays for haemagglutinating viruses and their antibodies.

In the particular case of the influenza and parainfluenza viruses, haemagglutination involves adsorption of a virus envelope glycoprotein (designated as either HA or HN

respectively) to mucoprotein receptors on the erythrocyte surface (Lazarowitz et al., 1971, 1973; Stanley and Haslam, 1971; Klenk et al., 1972; Scheid et al., 1972; Skehel, 1972; Scheid and Choppin, 1973). Viral neuraminidases, a separate envelope glycoprotein, NA, of the influenza viruses but a component of the HN glycoprotein of parainfluenza viruses, elute virus from the erythrocyte surface by hydrolytic cleavage of the glycoside linkage joining neuraminic acid to galactose with the release of N-acetyl neuraminic acid (Gottschalk, 1959; Scheid et al., 1972; Laver, 1973). This phenomenon of adsorption and elution has been used extensively with influenza viruses as a preliminary step in purification (Ada and Perry, 1956; Hoyle et al., 1961; Laver, 1973).

During the course of a study of the protein components of human parainfluenza type 2 viruses (Cowley and Barry, 1983), it occurred to us that adsorption of viruses to erythrocytes with immediate processing of the virus–red cell aggregates by PAGE might provide a simple analytic procedure for characterizing virus proteins. In our preceding studies we had routinely purified [^{35}S]methionine-labelled virus by conventional procedures, involving differential centrifugation and density gradient centrifugation. These methods ultimately produced virus of adequate concentration and purity for PAGE, but were both costly and time-consuming.

In this paper, we describe a simple preparative procedure for the identification and comparison of the [^{35}S]methionine-labelled structural proteins of influenza and parainfluenza viruses. The technique involves the adsorption of clarified tissue culture medium with a 0.5% v/v suspension of chicken or guinea-pig erythrocytes, subsequent dissociation of the virus–red cell aggregates and analysis directly by SDS–PAGE.

The results suggest that this procedure may have wide applicability for the analysis and comparison of the proteins of many other haemagglutinating viruses.

METHODS

Cells and viruses
Cynomolgus monkey embryonic kidney (MEK$_3$) cells and human parainfluenza virus (HPV) strains, HPV1–P$_1$1966; HPV2–P$_2$1972/5, P$_2$1972/6, P$_2$1980;HPV3–P$_3$1959, P$_3$1960, P$_3$1967, P$_3$1974; HPV4–P$_4$A1971, P$_4$A1972, P$_4$B1975, P$_4$B1976, were obtained from Dr. I.D. Gust, Fairfield Hospital for Communicable Diseases, Victoria, Australia. Madin–Darby canine kidney (MDCK) cells and influenza A virus (IAV) strains A/Ann Arbor/6/60 (H2N2), A/Queensland/6/72 (H3N2) and A/Hong Kong/123/77 (H1N1) have been used routinely by one of us (G.A.T.) for many years. HPV and IAV strains were propagated in MEK$_3$ and MDCK cells, respectively. Culture medium containing 1 µg/ml trypsin was used for the growth of HPV1 and IAV.

Erythrocyte preparation

Chicken and guinea-pig erythrocytes were collected and washed by standard methods (Palmer et al., 1975). Washed erythrocytes were suspended in 10 ml phosphate buffered saline (PBS), pH 7.3, and the packed cell volume (PCV) determined using a Clements micro-haemocrit centrifuge. Erythrocytes were diluted with PBS to give a 0.5% v/v suspension. Haemagglutination titrations were performed as described previously (Palmer et al., 1975; Shimokata et al., 1980).

Isotopic labelling of viruses

MEK_3 and MDCK cell monolayers in 9.6 cm^2 Petri dishes were infected with HPV and IAV strains, respectively, at approximately 1 plaque forming unit (PFU)/cell. At the onset of cytopathic effect (CPE), 16–18 h post-infection (HPI) for HPV-infected MEK_3 cells and 8 HPI for IAV infected MDCK cells, the culture medium was replaced with 1 ml of methionine-free medium 199 supplemented with 20 μCi/ml [^{35}S]methionine (1200 Ci/mmol). Cells were incubated at 37°C until 75–100% CPE had developed. Culture supernatants containing labelled virus were clarified at 10,000 × g for 20 min at 4°C and stored at –80°C.

Virus adsorption to erythrocytes

Aliquots (0.5 ml) of clarified virus-containing medium were added to 0.5 ml volumes of chicken or guinea-pig erythrocyte suspensions. IAV was adsorbed to chicken or guinea-pig erythrocytes for 1 h at 4°C while HPV was adsorbed to guinea-pig erythrocytes for 2 h at room temperature. Virus–erythrocyte suspensions were diluted to 10 ml with PBS, pelleted at 900 × g, 5 min at 4°C and twice washed with 10 ml PBS. The final virus–erythrocyte pellets were air dried, dissociated in 150 μl electrophoresis sample buffer (0.05 M Tris (hydroxymethyl) methylamine–HCl pH 6.8, 2% SDS, 12% 2-mercaptoethanol, 20% glycerol and 0.001% bromophenol blue) and boiled for 3 min. Chicken erythrocyte samples were aspirated vigorously through a 23-gauge needle to fragment the DNA.

Purification of HPV strains

Medium containing [^{35}S]methionine-labelled HPV strains was subjected to sucrose density gradient purification procedures as described previously (Cowley and Barry, 1983).

Polyacrylamide gel electrophoresis

Samples were analysed on either 12.5% linear or 7.5–15% gradient SDS–PAGE essentially following the procedure of Laemmli (1970). Proteins were visualised by staining with Coomassie brilliant blue and gels were fluorographed by the method described by Chamberlain (1979). Dried gels were exposed to Kodak X-Omat XRP-5 films at –80°C.

RESULTS

Standardizing conditions

The procedure outlined below involves the adsorption of radio-labelled virus to erythrocytes and the subsequent analysis of the virus–red cell aggregate by SDS–PAGE. To optimize the ratio of virus to red cells, and to minimize any distortion or interference effects attributable to large amounts of erythrocyte protein, experiments were carried out to determine both the efficiency of virus adsorption and the distribution of erythrocyte-derived protein in gradient polyacrylamide gels, as detected by Coomassie blue staining.

To determine the efficiency of adsorption, 0.05 ml aliquots of gradient purified [^{35}S]methionine-labelled HPV3 were added to microtitre plate wells containing a range of concentrations of guinea-pig red cells (0.03–8%), suspended in 0.05 ml vols. The mixtures were incubated for 2 h at room temperature, and the erythrocytes pelleted by centrifugation at 2,500 rpm, 10 min at 4°C. Unadsorbed [^{35}S]methionine remaining in the supernatant was subtracted from the total counts added and plotted against guinea pig erythrocyte concentration (Fig. 1). More than 50% of the total virus was adsorbed by red cell suspensions of 0.125% v/v, although concentrations in excess of 2% v/v were required to achieve more than 90% adsorption.

The distribution and abundance of erythrocyte proteins was established by subjecting a range of concentrations (0.06–2%) to SDS–PAGE together with a range of standard mol. wt. marker proteins (Fig. 2). Present in all samples is a large amount of protein of mol. wt. approx. 15 kd, representing the α- and β-globin molecules, and which might affect the migration of virion polypeptides of this size; the number and concentration of other erythrocyte proteins is relatively small. As neither IAV or HPV contain structural proteins of mol. wt. lower than 25 kd, the presence of red cell proteins in the gel was not considered to be a problem at concentrations of 0.5% or
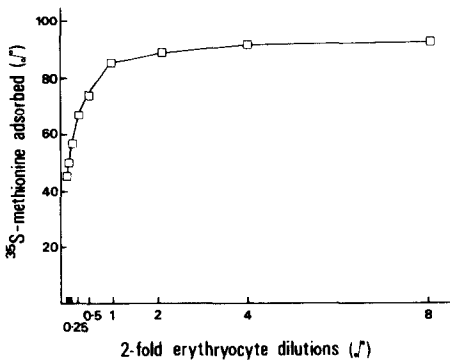

Fig. 1. Adsorption of [^{35}S]methionine-labelled HPV3-P$_3$1974 (160 HA U/ml) to 2-fold dilutions of guinea-pig erythrocytes.
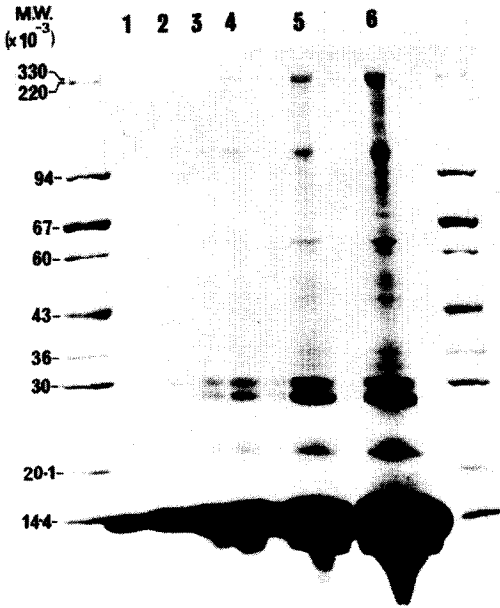
Fig. 2. Effect of increasing concentrations of guinea-pig erythrocytes on band resolution in SDS–polyacry-lamide gel. Erythrocyte concentrations, 0.0625% (lane 1), 0.125% (lane 2), 0.25% (lane 3), 0.5% (lane 4), 1.0% (lane 5) and 2.0% (lane 6) were electrophoresed together with the standard mol. wt. marker proteins, thyroglobulin (330 kd), ferritin (220 kd), phosphorylase b (94 kd), bovine serum albumin (67 kd), catalase (60 kd), ovalbumin (43 kd), lactate dehydrogenase (36 kd), carbonic anhydrase (30 kd), trypsin inhibitor (20.1 kd) and α-lactalbumin (14.4 kd) in a 7.5–15% gradient gel and stained with Coomassie brilliant blue as described in Materials and methods.

less. In the experiments reported below, red cells at a concentration of 0.5% v/v were used for adsorption and SDS–PAGE analysis.

Influenza virus proteins

Uninfected MDCK cells and cells infected with IAV strains A/Ann Arbor/6/60, A/Queensland/6/72 and A/Hong Kong/123/77 were incubated for 8 h, at which time the culture medium was replaced with methionine-free 199 medium supplemented with 20 μCi/ml [^{35}S]methionine. At the development of extensive cytopathic change, the labelling medium was clarified and adsorbed with 0.5% v/v suspension of chicken or guinea-pig erythrocytes for 1 h at 4°C. The virus–erythrocyte aggregates were dissociated and analysed by SDS–PAGE (Fig. 3). The polypeptide profiles of the three influenza virus strains were identical regardless of whether (a) chicken or (b) guinea pig erythrocytes were used for virus adsorption (Fig. 3, lanes 2, 3 and 4). However, as chicken erythrocytes contain DNA, such samples require vigorous aspiration to facilitate easy loading. The influenza virion polypeptides P$_1$, P$_2$, P$_3$, HA, HA1, RNP, NA, M and HA2 have been designated according to the standard
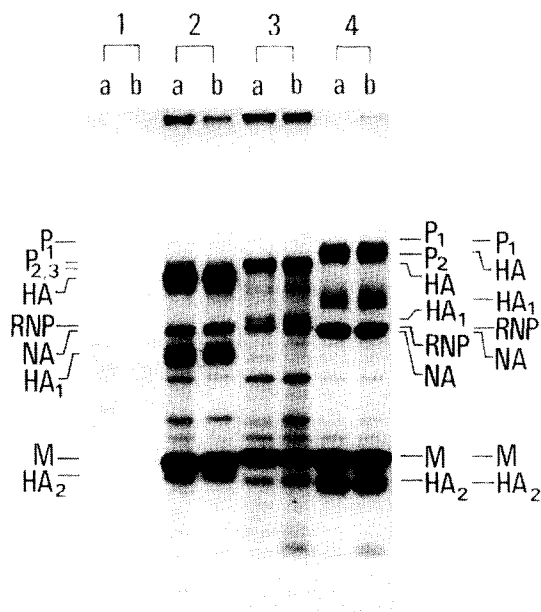
Fig. 3. Comparison of the polypeptide composition of [³⁵S]methionine-labelled influenza virus strains adsorbed to chicken or guinea-pig erythrocytes. Culture medium containing the IAV strains A/Ann Arbor/6/60 (lane 2), A/Queensland/6/72 (lane 3) and A/Hong Kong/123/77 (lane 4) was adsorbed to (a) chicken and (b) guinea-pig erythrocytes as described in Materials and methods. Uninfected MDCK culture medium (lane 1a, b) was similarly adsorbed as a control. Samples were analysed on a 7.5–15% gradient SDS–polyacrylamide gel. The designations of the polypeptides of A/Ann Arbor/6/60 are to the left, A/Queensland/6/72 and A/Hong Kong/123/77 to the right and far right, respectively.

nomenclature (see reviews by Schulze, 1973; White, 1974; Choppin and Compans, 1975). The haemagglutinin polypeptide, HA, and its cleavage products, HA1 and HA2, vary considerably in migration between the three serologically distinct strains, A/Ann Arbor/6/60 (H2N2) (Fig. 3, lane 2), A/Queensland/6/72 (H3N2) (Fig. 3, lane 3) and A/Hong Kong/123/77 (H1N1) (Fig. 3, lane 4). Some polypeptides seen between the NA and M polypeptides of all three influenza virus strains may correspond to proteins that were also adsorbed from the culture medium of uninfected MDCK cells by both chicken and guinea-pig erythrocytes (Fig. 3, lane 1a, b).

Parainfluenza virus proteins

To investigate the possibility that the protein composition of other haemagglutinating virus groups could be examined after erythrocyte adsorption a number of HPV strains were analysed. MEK₃ cells infected with HPV type 1, 2, 3, 4A and 4B strains were labelled with [³⁵S]methionine from 16 HPI until significant cytopathic change was evident. Clarified culture medium was adsorbed with a 0.5% v/v suspension of guinea-pig erythrocytes for 2 h at room temperature, and the dissociated virus–ery-

throcyte aggregates analysed by SDS–PAGE are shown in Fig. 4. Uninfected MEK$_3$ culture medium was similarly adsorbed and run as a control (Fig. 4a). As constant amounts of [^{35}S]methionine per well were loaded the apparent background of adsorbed non-viral polypeptides was significantly lower in virus-adsorbed preparations (Fig. 4, b–m) compared to the control (Fig. 4a). The control gel indicates that a major polypeptide of mol. wt. 67 kd has been adsorbed from uninfected MEK$_3$ cell medium and migrated in the same position as the polypeptide designated NP for HPV type 2, 3 and 4. The possibility that certain cell proteins may have high binding affinities for erythrocytes must always be considered.

The polypeptides of HPV1 (Fig. 4b) are designated according to the nomenclature adopted for the serologically related Sendai virus (see review by Choppin and Compans, 1975); HPV2 (Fig. 4, c, d, e) are designated according to Cowley and Barry (1983), HPV3 (Fig. 4,f, g, h, i) and HPV4 (Fig. 4,j, k, l, m) on the basis of the results of Guskey and Bergtrom (1981) and Cowley and Barry (unpubl. obs.).
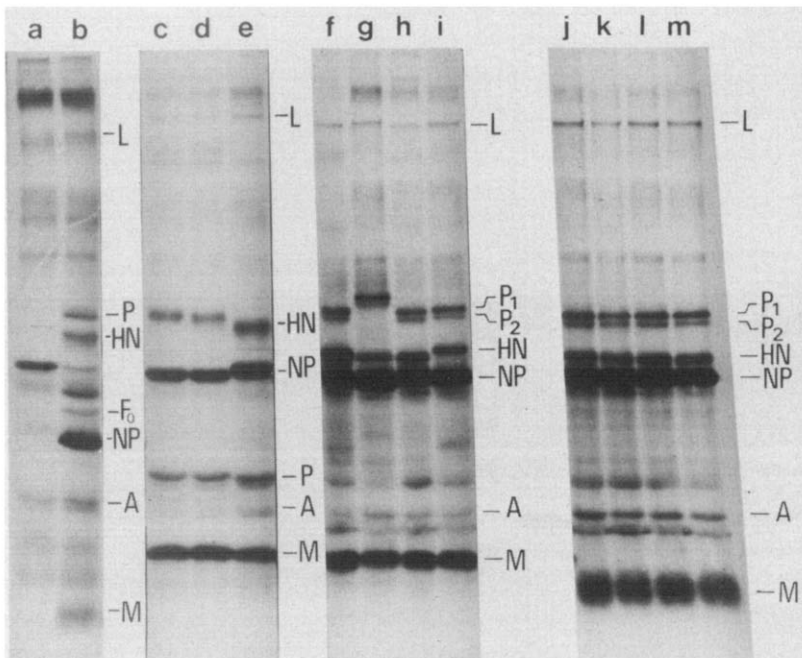

Fig. 4. Analysis of the polypeptide composition of HPV type 1, 2, 3, 4A and 4B strains adsorbed to guinea-pig erythrocytes. Uninfected MEK$_3$ culture medium (a) and culture medium containing a number of [^{35}S]methionine-labelled HPV strains, HPV1-P$_1$1966 (b); HPV2-P$_2$1972/5 (c), P$_2$1972/6 (d), P$_2$1980 (e); HPV3-P$_3$1960 (f), P$_3$1959 (g), P$_3$1967 (h), P$_3$1974 (i); and HPV4-P$_4$A1971 (j), P$_4$A1972 (k), P$_4$B1975 (l), P$_4$B1976 (m) were adsorbed to guinea-pig erythrocytes and virus-erythrocyte complexes were subjected to electrophoresis on a 12.5% SDS–polyacrylamide gel as described in Materials and methods.

Comparison of sucrose gradient purified and erythrocyte-adsorbed HPV

To demonstrate the effectiveness of the erythrocyte adsorption procedure for viral protein analysis, HPV-erythrocyte complexes were run in parallel with virus preparations that had been purified by equilibrium centrifugation on 15–60% w/v sucrose density gradients (Fig. 5). Clarified MEK_3 cell culture medium containing [^{35}S]methionine-labelled HPV type 2, 3 and 4 strains, HPV2-$P_2$1972/6 (Fig. 5, lane 1), $P_2$1980 (Fig. 5, lane 2); HPV3-$P_3$1959 (Fig. 5, lane 3), $P_3$1967 (Fig. 5, lane 4), $P_3$1974 (Fig. 5, lane 5) and HPV4-P_4A 1971 (Fig. 5, lane 6) was obtained. Samples of this medium were either adsorbed directly with 0.5% v/v guinea-pig erythrocyte suspensions or subjected to concentration and purification on 15–60% w/v sucrose density gradients centrifuged at 200,000 $\times$ g for 3 h, and then analysed by SDS–PAGE. All virus polypeptides observed in sucrose gradient purified preparations (Fig. 5a) were identical in their migrational properties and were present in identical proportions to
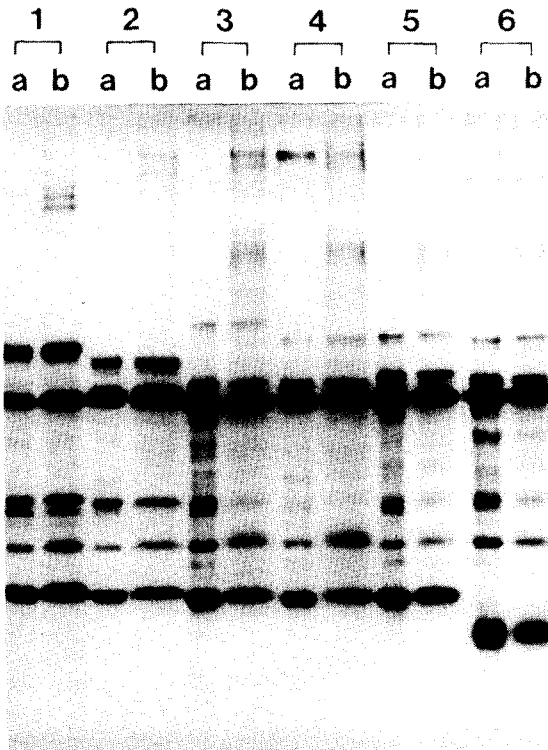

Fig. 5. Comparison of the polypeptide profiles of [^{35}S]methionine-labelled HPV strains purified on sucrose density gradients or adsorbed to guinea-pig erythrocytes. HPV type 2, 3 and 4 strains HPV2-$P_2$1972/6 (lane 1), $P_2$1980 (lane 2); HPV3-$P_3$1959 (lane 3), $P_3$1967 (lane 4), $P_3$1974 (lane 5); and HPV4-P_4A 1971 were (a) purified by equilibrium centrifugation on 15–60% w/v sucrose density gradients or (b) adsorbed to guinea-pig erythrocytes as described in Materials and methods. Samples were analysed on a 12.5% SDS–polyacrylamide gel.

polypeptides derived from erythrocyte adsorbed samples (Fig. 5b). However, some minor loss in band resolution and some additional high molecular weight material (probably representing incompletely dissociated protein aggregates) was observed in virus–adsorbed erythrocyte samples.

DISCUSSION

In this study the structural polypeptides of stains of IAV and HPV types 1, 2, 3 and 4 have been analysed by the simple procedure of virus adsorption to erythrocytes and SDS–PAGE of virus–erythrocyte aggregates. The method is an adaptation of the conditions of a standard HA titration (Shimokata et al., 1980), whereby equal 0.5 ml volumes of clarified culture medium containing [^{35}S]methionine labelled virus and 0.5% chicken or guinea pig erythrocytes are mixed and allowed to adsorb for 1–2 h; IAV-red cell mixtures are held at 4°C to prevent elution.

Although preliminary experiments indicated that these conditions were satisfactory, it was considered desirable both to titrate virus adsorption against red cell concentration (Fig. 1) and to ensure that the presence of unlabelled erythrocyte proteins in the gel did not cause severe band distortion (Fig. 2). In both respects it was found, using HPV3 and guinea-pig red cells, that 0.5% v/v was the most suitable concentration. At this concentration 80% adsorption was achieved without overloading the gel with extraneous proteins; higher concentrations of red cells provide a relatively small incremental increase in adsorption but are likely to introduce distortion. The results obtained for IAV (Fig. 3) using both chicken or guinea pig cells suggest that a concentration of 0.5% v/v is suitable also for these viruses.

When the IAV A/Ann Arbor/6/60 (H2N2), A/Queensland/6/72 (H3N2) and A/Hong Kong/123/77 (H1N1) were adsorbed to either chicken or guinea-pig erythrocytes and subjected to electrophoresis all of the virus structural proteins, namely P. HA, HA1, HA2, RNP, NA and M that had previously been described for influenza viruses (Schulze, 1973; White, 1974; Choppin and Compans, 1975) were clearly resolved (Fig. 3). No observable difference in the migrational properties or relative proportions of the virus structural proteins was observed when either chicken (Fig. 3a) or guinea-pig erythrocytes (Fig. 3b) were used for virus adsorption.

Similar findings have been obtained for HPV in that the HA–PAGE procedure provides a simple concentration–purification step with resolution of structural proteins comparable to that obtained with sucrose gradient purified virus (Figs. 4 and 5). The human parainfluenza viruses have received scant attention in the past because they are usually associated with poor yield in tissue culture and extreme lability (Kingsbury, 1977; Goswami and Russell, 1982), and thereby provide good models for testing the general applicability of the red-cell adsorption method. In other studies we have established (Cowley and Barry, 1983; unpubl. obs.) that all HPV comprise at least 6 major structural polypeptides which have been identified in Fig. 4 as L, P. HN, NP, F and M. All HPV polypeptides are not only resolved readily by the red-cell

adsorption procedure, but within serotype 2 (Fig. 4, lanes c–e) and serotype 3 (Fig. 4, lanes f–i) the previously reported differences in the gel migration of the HN and NP polypeptides are apparent (Cowley and Barry, 1983). Since such differences may reflect genetic diversity within HPV serotypes, we believe that the red-cell adsorption method could be of general usefulness in probing the molecular epidemiology of HPV, and of a wide range of other haemaggluting viruses.

ACKNOWLEDGEMENTS

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