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Avian Infectious Bronchitis Virus Structural Polypeptides: Effect of Different Conditions of Disruption and Comparison of Different Strains and Isolates

By

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With 7 Figures

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Summary

Variations in the conditions used for disruption of purified virus involving differences in heat treatment and reducing agent concentration produced little affect on the polypeptide profiles of the Massachusetts 41 (M41) strain of avian infectious bronchitis virus obtained by polyacrylamide gel electrophoresis.

Comparisons of the structural polypeptides of 12 IBV isolates, consisting of M41, six serologically related viruses and representatives of five other serotypes, showed that the viruses could be placed in three groups on the molecular weights of the major glycopolypeptides. These were 31,000 and 86,000 for M41, the six related viruses and the serologically distinct SE17; 27,000 and 89,000 for Iowa 97 and Holte and 27,000 and 91,000 for Connecticut and T strain.

Introduction

Examination of the structural polypeptides of different coronaviruses have produced results indicating as little as three or as many as 16 polypeptides although in all cases comparable polypeptides to those of other viruses were detected (for review see 19). Studies on the structural polypeptides of avian infectious bronchitis virus (IBV) show considerable variation in the results obtained. Bingham (4) and Alexander and Collins (2, 7) have produced basically similar results, suggesting that IBV consists of 14—16 polypeptides, but MacNaughton and Madge (15) concluded there were only seven structural polypeptides. This latter report is more in keeping, both in number and size, with the polypeptides reported for other coronaviruses.

Several suggestions have been put forward to account for the large number of polypeptides seen in the earlier studies of IBV. Alexander and Collins (2) studied exhaustively the purification of the virus and their work indicates that

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the problem was not merely one of failure to produce sufficiently purified virus. Sturman (20) suggested that studies using only polypeptide staining of gels may detect unincorporated host polypeptides which may be attached to virions. However almost identical profiles have been obtained using stained or ^{3}H-leucine labelled polypeptides (2). Sturman (20) has also demonstrated that different concentrations of reducing agent and heating during preparation of coronavirus A59 polypeptides affected the number present and suggested that artifacts produced in this way may account for some of the polypeptides seen in studies of IBV. MacNaughton and Madge (15) have also stressed that the effect of harsh conditions during treatment of virus was to increase the number of polypeptides seen after polyacrylamide gel electrophoresis (PAGE) and by such treatment were able to produce polypeptide profiles consisting of more than 12 bands. In the present study we have examined the effects of the different conditions described by these authors on the polypeptide profile of the Massachusetts 41 strain of IBV.

Many serotypes of IBV have been identified (8, 13) and strains may differ considerably in other properties (10). Comparative studies of the polypeptides of different IBV strains have been limited to one of four strains (two serotypes) by Bingham (4) and three of the same strains (two serotypes) by MacNaughton and Madge (15). In both studies no significant differences were reported between the polypeptide profiles of the different strains. In the present study we have used twelve strains of IBV representing 6 serotypes, vaccinal strains and field isolates for polypeptide analysis.

Table 1. *Properties of avian infectious bronchitis strains and isolates*

Virus	No. egg passages	Corona		Sero-type (13)	Group titre (6)
		Study A (10)	Study B[a]		
Massachusetts 41	4 (402 bird passages)	+[b]	++++	1	L[c]
Connecticut	9	+	++++	2	L
SE 17	7	ND	+++	3	ND
Holte	18	++	++	4	ND
Iowa 97	Not known	ND	++	5	ND
T	3	+++	+++	8	H
Beaudette	>250	−	+*	1[d]	L
H 52 (vaccine)	>52	ND	+++	1[d]	H
H 120 (vaccine)	>120	+++	+++	1[d]	H
927 (field isolate)	6	−*	++*	1[d]	L
381 (field isolate)	2	ND	ND	1[d]	ND
563 (field isolate)	2	ND	++	1[d]	ND

[a] Collins and Alexander unpublished results

[b] Visual estimations of corona
− : corona rarely seen on particles
++++ : complete corona on most particles
* : atypical projections

[c] L: low group titre, H: high group titre (6, 10)

[d] These strains and isolates were not specifically used by Hopkins (13) but are related to M41

ND—Not determined

Materials and Methods

Virus Strains

All IBV strains were obtained from Dr. C. D. Bracewell, Central Veterinary Laboratory, Weybridge, their histories have been described (6). The strains used in the present study and some of their reported properties are listed in Table 1.

Viruses were grown in the allantoic cavity of 9 or 10-day-old embryonated fowls' eggs by inoculation of 10^3—10^4 EID_{50} of virus in 0.1 ml. Incubation was at 37° C for 24—72 hours depending on the strain, live embryos were then chilled overnight at 4° C and the allantoic-amniotic fluids harvested.

Virus Purification

The infectious allantoic-amniotic fluid was clarified by centrifugation at $2000 \times g$ for 20 minutes. Virus was then pelleted by centrifugation at $20{,}000 \times g$ for 30 minutes and resuspended at about 100-fold concentration in 0.01 M Tris-HCl buffer pH 6.5. The concentrated virus was then recentrifuged for five minutes at $2000 \times g$, to remove any large aggregates, and the supernatant placed on an 18 ml 20—55 per cent w/w sucrose gradient made in 0.01 M Tris-HCl buffer pH 6.5 and centrifuged at $44{,}000 \times g$ for 30 minutes. The virus peak from this rate zonal gradient was then applied to a similar sucrose gradient but this time centrifuged for 18 hours at $70{,}000 \times g$. The purified virus was then centrifuged to a pellet once more and resuspended in either 0.01 M phosphate buffer pH 7.2 or 0.01 M Tris HCl buffer pH 7.2 depending on the electrophoretic system to be used. All centrifugation steps were done at 4° C.

Polyacrylamide Gel Electrophoresis (PAGE)

In experiments to test the effects of different preparative conditions on the polypeptide profiles of IBV purified M41 virus was disrupted with sodium dodecylsulphate (SDS) and reducing agent (2-mercaptoethanol or dithiothreitol) at the specified concentrations. For comparative PAGE of different strains of IBV two per cent w/v SDS, two per cent v/v 2-mercaptoethanol and boiling for two minutes was used to disrupt the virus which was then made five per cent sucrose and 0.001 per cent bromophenol blue.

Disrupted virus samples containing about 100 μg of protein in 0.05 ml were layered on each 80×7 mm cylindrical polyacrylamide gel containing 7.5, 10 or 13 per cent w/v acrylamide at a constant ratio of acrylamide:bisacrylamide of 37.5:1 by weight. All other conditions of PAGE were as described for the phosphate (continuous) system (1) or the discontinuous system (3).

Polypeptides were detected by staining gels with 0.1 per cent Coomassie brilliant blue and glycopolypeptides by staining with Schiff's reagent as described (2).

The molecular weights of the polypeptides were estimated by comparison of the migration following SDS-PAGE with standards of known molecular weight. The standards used were: bovine serum albumin, phosphorylase A, and ribonuclease. Using these standards estimations of the molecular weights of the polypeptides of Newcastle disease virus (Ulster strain), which was usually run in parallel, were similar to the values reported by Moore and Burke (17).

Protein Estimation

Protein was estimated by the method of Lowry *et al.* (14) using bovine serum albumin as a standard.

Results

Buoyant Densities of Strains

Providing virus was treated by centrifugation at $2000 \times g$ for 5 minutes after concentration and initial purification and immediately before isopycnic sucrose density centrifugation, although broad-based, only one peak of virus activity was

apparent. All strains were given at least two runs and the peak of virus activity was found at about 1.17 g/cm^3 for all strains except Beaudette and M41 which were marginally denser with average density of 1.18 g/cm^3 (average of three runs for each strain) and Connecticut which consistently produced a peak of virus at 1.16 g/cm^3 (three runs).

Polypeptide Analysis by PAGE

Two systems of PAGE were used: a continuous phosphate-buffered method and a discontinuous Tris-glycine buffered method. Except for minor differences in some polypeptide migration rates the results obtained were identical for both systems although usually better resolution could be obtained with the discontinuous system.

Throughout these studies no differences were detected if dithiothreitol was substituted for mercaptoethanol.

Analysis of polypeptides was done using 7.5, 10 and 13 per cent acrylamide gels. Molecular weight estimations showed close agreement for each gel concentration and those quoted below are average figures for at least one run at each of the three concentrations.

Analysis of purified M41 virus by PAGE and staining with Coomasie brilliant blue revealed as many as 30 polypeptide bands. A typical polypeptide profile of M41 obtained by SDS-PAGE under reduced conditions is shown in Figure 1 with

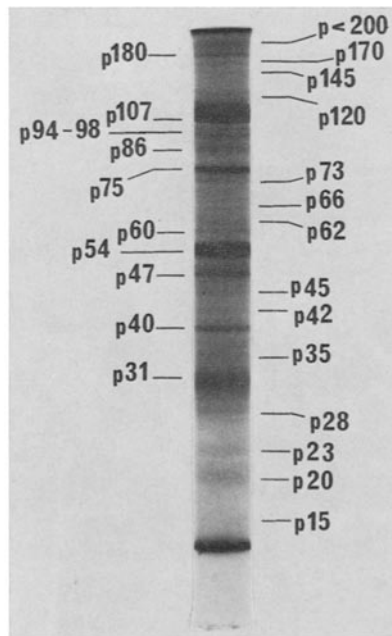

Fig. 1. Polypeptide profile of IBV strain Massachusetts 41. Purified virus was disrupted with two per cent SDS and two per cent mercaptoethanol and boiled for two minutes prior to separation by discontinuous SDS-PAGE on a 10 per cent acrylamide gel and staining with Coomasie brilliant blue. The most prominent bands are labelled and the number represents the estimated molecular weight $\times 10^{-3}$. The eleven bands with labels on the left of the gel were arbitrarily nominated as the major polypeptides

the 25 most prominent polypeptides labelled. The majority of the polypeptide bands represented only a very small proportion of the total protein and could be considered minor, while based on the proportion of total protein and retrospectively on the results obtained in the present study 11 were nominated as major polypeptides: p180, p107, p98, p94, p86, p75, p60, p54, p47, p40 and p31 (Fig. 1).

All the major polypeptide bands were seen consistently with the exception of p107 and p75 which, although always present in different gels, varied greatly in quantity from preparation to preparation of virus. The presence or absence of large amounts of these polypeptides did not correlate with any changes in the proportions of other polypeptides and variation of the two was seen in preparations of seven of the 12 IBV strains studied.

Effects of Conditions of Preparation for PAGE on the Migration of IBV-M41 Polypeptides

Marked differences were recorded between the polypeptide patterns of M41 run under reduced (Fig. 2a, c and e) and non-reduced (Fig. 2b and d) conditions. In the absence of reducing agent many of the minor bands and the major bands

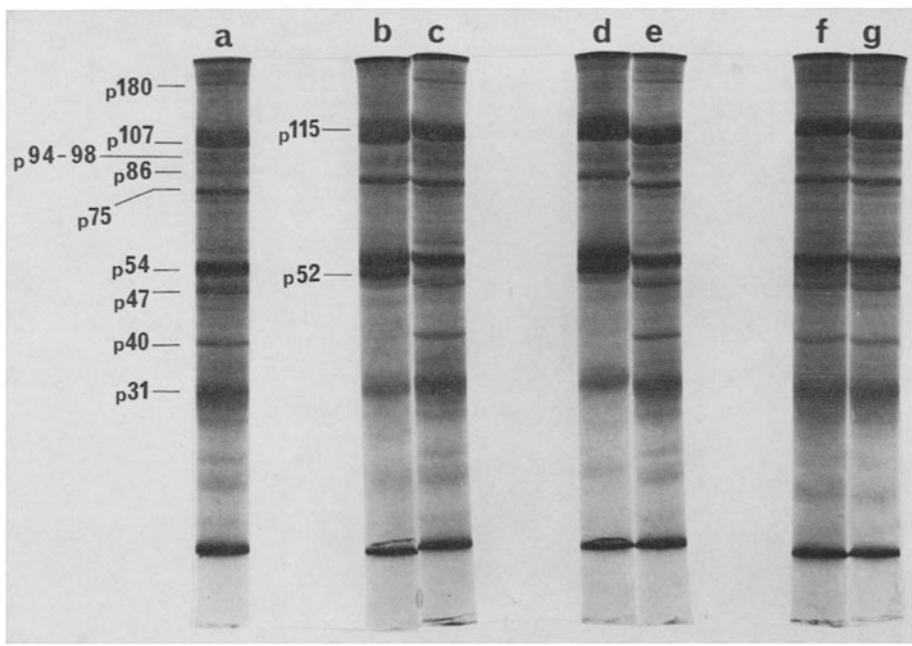

Fig. 2. Effect of different preparative conditions on the polypeptide profile of IBV strain Massachusetts 41. Prior to discontinuous PAGE on 10 per cent polyacrylamide gels and staining with Coomasie brilliant blue a purified preparation of M41 virus was disrupted under the following conditions: *a* two per cent SDS, two per cent mercaptoethanol at room temperature; *b* two per cent SDS, no mercaptoethanol, boiled for two minutes; *c* two per cent SDS, two per cent mercaptoethanol, boiled for two minutes; *d* two per cent SDS, no mercaptoethanol, held at 37° C for 10 minutes; *e* two per cent SDS, two per cent mercaptoethanol, held at 37° C for 10 minutes; *f* dialysed overnight against one per cent SDS and 0.1 per cent mercaptoethanol, boiled for two minutes before and after dialysis; *g* dialysed overnight against one per cent SDS and 0.1 per cent mercaptoethanol no boiling

p40 and p47 seen in reduced gels were absent, while additional major bands p115 and p52 (possibly two polypeptides) were present.

The polypeptide pattern obtained on gels of M41 following overnight dialysis against 1 per cent SDS and 0.1 per cent mercaptoethanol was unaffected by boiling for two minutes before and after dialysis (Fig. 2f and g). In gels of virus prepared this way all the polypeptides seen in gels reduced with 2 per cent mercaptoethanol were present but in addition low levels of p115 and p52 were detected which presumably indicates incomplete reduction.

The effects of different concentrations of mercaptoethanol on the polypeptide profile of M41 were also examined (Fig. 3). No differences were seen when 2 or 5 per cent mercaptoethanol was used but at higher concentrations, 10 and 20 per cent, a high proportion of the protein failed to enter the gels and a marked decrease in the migration of the polypeptides was seen. However, even in gels run in the presence of 20 per cent mercaptoethanol all the major polypeptides seen at lower concentrations were discernible.

Comparison of the Polypeptides of Different Strains of IBV

Twelve strains of IBV (Table 1) were purified and compared by PAGE on 7.5, 10 and 13 per cent gels. Staining with Schiff's reagent was used to detect glycosylation and overstaining with Coomassie brilliant blue enabled precise

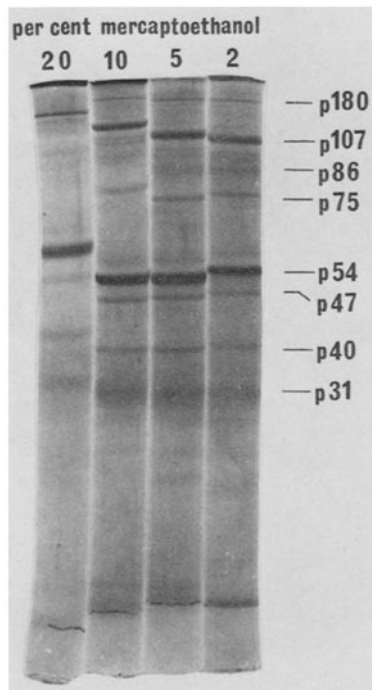

Fig. 3. Effect of different concentrations of mercaptoethanol on the polypeptide profile of IBV strain Massachusetts 41. Prior to discontinuous PAGE on 10 per cent polyacrylamide gels a purified preparation of M41 was treated with two per cent SDS and two, five, 10 or 20 per cent mercaptoethanol, as indicated, and boiled for two minutes. Polypeptides were stained with Coomasie brilliant blue. The origin is at the top

location of the glycopolypeptides. Typical polypeptide profiles are shown in Figures 4—7.

The two sets of gels stained with Coomasie blue shown in Figure 4 (a and b) are 10 and 13 per cent acrylamide gels of different preparations of the same six IBV strains. The quantitative variability of p107 and p75 can be most noticeably seen for the Holte, T and Connecticut strains. Apart from these variations, which were not strain specific, the major differences in the polypeptide patterns of the various strains were associated with the glycopolypeptides. On most gels stained with Schiff's reagent (Fig. 5) four bands were seen, two of these stained poorly but were similar for all strains and corresponded to polypeptides p107 and p54. On some gels an additional fifth area of faint Schiff's staining was detected which corresponded to a polypeptide of 65,000—70,000 molecular weight. All strains showed two major glycopolypeptides but the migration rates varied with strains. Of the six strains shown in Figures 4 and 5, glycopolypeptide p31 was present in gels of M41 and Beaudette but in gels of the other four strains the lower molecular weight major glycopolypeptide migrated with an apparent molecular weight of 27,000. Detection of differences in the migration of the higher molecular weight major glycopolypeptide were complicated by the presence of non-glycosylated polypeptides in the 94,000 molecular weight area but careful analysis of 7.5 per

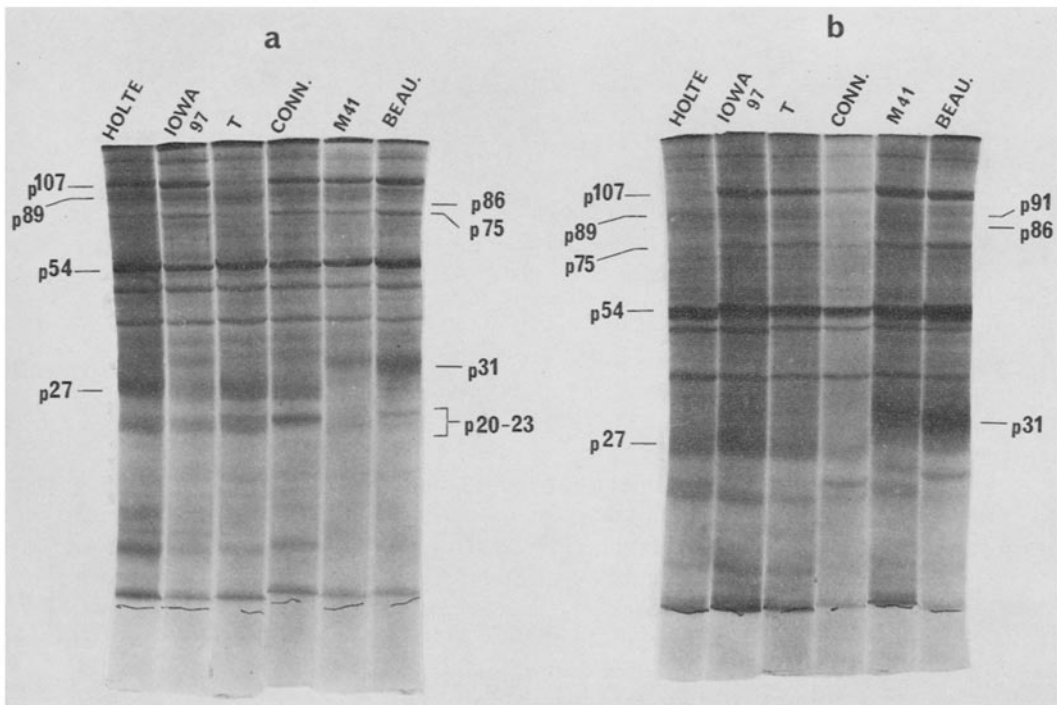

Fig. 4. Comparison of the polypeptide profiles of six strains of IBV. Each purified preparation of the indicated strains was treated with two per cent SDS, two per cent mercaptoethanol and boiled for two minutes prior to discontinuous PAGE. *a* 13 per cent acrylamide gels stained with Coomasie brilliant blue; *b* 10 per cent acrylamide gels stained with Coomasie brilliant blue

cent gels stained with Schiff's reagent gave molecular weight values for this glycopolypeptide of 86,000 for Beaudette and M41, 89,000 for Iowa 97 and Holte and 91,000 for T and Connecticut.

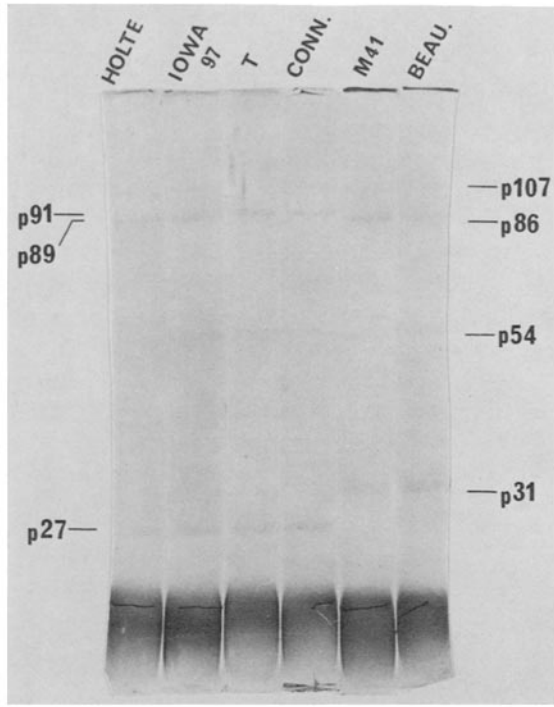

Fig. 5. Comparison of the glycopolypeptides of six strains of IBV. Conditions were as for those gels in Figure 4 except that 7.5 per cent acrylamide was used and gels were stained with Schiff's reagent for glycopolypeptides

SDS-PAGE of the viruses serologically related to M41: H120, H52, 927, 563 and 381 and the serologically distinct SE17 produced polypeptide profiles similar to that of M41 (Fig. 6, 563 and 381 not shown). However, some variations in addition to those of p107 and p75, were seen between the different strains. In particular H120 showed both quantitative differences and a polypeptide of 49,000 molecular weight which was not detected in other viruses (Fig. 6). However SDS-PAGE followed by Schiff's staining revealed the major glycopolypeptides of all six viruses to be of 86,000 and 31,000 molecular weight (Fig. 7, 381 not shown).

In addition to the differences in glycopolypeptides of the 12 strains some qualitative and quantitative variations were observed in the minor polypeptide bands, particularly those with migration rates faster than the 27,000/31,000 molecular weight glycopolypeptide (Figs. 4—6). Although all strains showed at least one polypeptide of molecular weight in the range 20,000—23,000 and at least one other at lower molecular weight, some strains had characteristic polypeptides in this area. For example polypeptide profiles of strain Connecticut consistently showed a prominent polypeptide of about 22,000 molecular weight. However, for

most viruses, these low molecular weight polypeptides were frequently indistinct and variable in migration and the apparent differences did not indicate further polypeptide groups amongst the strains tested than those distinguishable by glycopolypeptide analysis.

Grouping of the virus strains by the molecular weights of the major glycopolypeptides is summarized in Table 2.

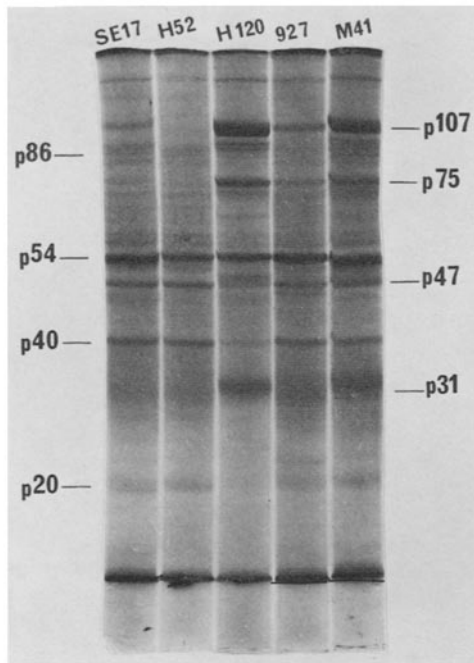

Fig. 6. Comparison of the polypeptides of five strains of IBV. Conditions were as for those gels in Figure 4b

Table 2. *The major glycopolypeptides of avian infectious bronchitis virus strains and isolates*

Glycopolypeptide group	Virus	Serotype	Molecular weights of major glycopolypeptides $\times 10^{-3}$	
	Massachusetts 41	1		
	Beaudette	1		
	H120	1		
1	H 52	1	86	31
	927	1		
	381	1		
	563	1		
1	SE 17	3	86	31
2	Holte	4	89	27
	Iowa 97	5	89	27
3	Connecticut	2	91	27
	T	8	91	27

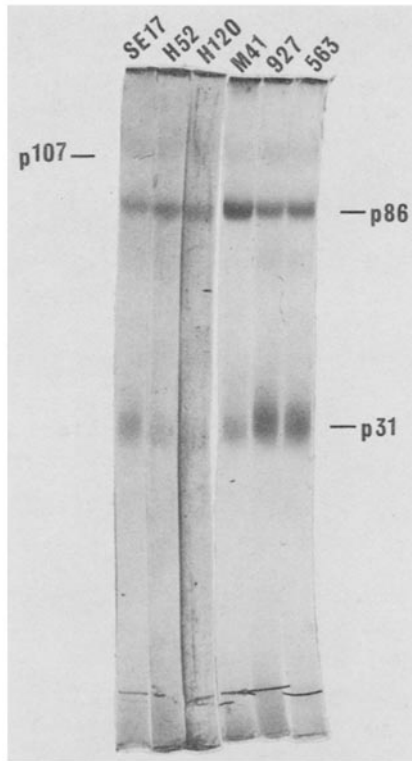

Fig. 7. Comparison of the glycopolypeptides of six strains of IBV. Purified virus preparations were treated with two per cent SDS and two per cent mercaptoethanol and boiled for two minutes prior to continuous (phosphate) SDS-PAGE on 10 per cent acrylamide gels and staining with Schiff's reagent

Discussion

Early reports of IBV structural proteins examined by SDS-PAGE (2, 4, 7) indicated the presence of 14—16 polypeptides, these findings contrasted greatly with studies of other coronaviruses which detected only 4—7 structural polypeptides (9, 11, 12, 18, 20). However a report by MacNaughton and Madge (15) suggested only seven polypeptides for IBV. Several reasons for the differences in the number of polypeptides detected in early studies of IBV and those of other coronaviruses have been put forward. Failure to obtain purified virus and differences between radioisotope labelled and stained polypeptide patterns were discounted by work by Alexander and Collins (2). In the present study we have examined the different preparative conditions reported to cause variation in polypeptide pattern for IBV and coronavirus A59 (15, 20) but have shown no differences in the number of polypeptides detected. In contrast to the results with A59 (20) our results with M41 showed that profiles of reduced and non-reduced polypeptides were different and that both were unaffected by temperature. IBV polypeptides exhibited neither association at elevated temperatures nor incomplete dissociation or metastability at low temperatures.

The large differences in the number of polypeptides reported in other studies with IBV and those in the present study (as many as 30) cannot be explained by differences in virus purification nor by different conditions during disruption of the purified virus. The majority of the polypeptides detected in the present study represented very small proportions of the total protein and may simply not have been detected in other studies. MACNAUGHTON and MADGE (15) reported the molecular weights of the seven polypeptides they detected by SDS-PAGE of IBV as: 130,000, 105,000, 97,000, 82,000, 74,000, 51,000 and 33,000 which show a close similarity to the molecular weights of seven of the eleven major polypeptides seen in the present study (Fig. 1). However, identification of the glycosylated polypeptides produced quite different results, MACNAUGHTON *et al.* (16) reporting the molecular weights of the glycosylated polypeptides as 130,000, 105,000, 82,000 and 74,000 compared to our estimates of 107,000, 86,000—91,000, 54,000 and 27,000—31,000. BINGHAM (4) reported glycopolypeptides of 180,000, 130,000, 83,000, 37,000 and 14,000. As all three studies have used staining with Schiff's reagent to detect glycosylation which does not always produce particularly strong staining it may well be that definitive identification of the glycosylated polypeptides awaits the use of radioisotopes.

Although throughout the present study a consistent polypeptide pattern was seen for IBV considerable quantitative variations in the 107,000 and 75,000 molecular weight polypeptides were detected. These were not strain specific but varied from preparation to preparation. Other polypeptides were unaffected by the amounts of p107 and p75 detected which suggests that these polypeptides were not precursors of other polypeptides but that in some preparations of virus they were absent. MACNAUGHTON *et al.* (16) showed that their comparable polypeptides of 105,000 and 74,000 molecular weight were surface polypeptides which could be removed by bromelain. It may be that in our studies on occasions preparative conditions were such that these polypeptides were removed, either physically or chemically, from the virus surface. However no morphological evidence was obtained to suggest that this had occurred.

Other comparisons of IBV strains have revealed no obvious differences in polypeptide profiles (4, 15). However in the present study of 12 IBV strains we were able to distinguish three groups of viruses based on the migration of the major glycopolypeptides. All the strains tested of the Massachusetts serotype: Beaudette, M41, the vaccinal strains H120 and H52, the British field strains 927, 563 and 381, and the serologically distinct SE17 strain had glycopolypeptides of molecular weight 31,000 and 86,000. Connecticut and T had major glycopolypeptides of molecular weight 27,000 and 91,000 while strains Iowa 97 and Holte had 27,000 and 89,000 molecular weight glycopolypeptides. Apart from the finding that all viruses of serotype 1 had similar major glycopolypeptides there was no relationship between the glycopolypeptide groups and any of the properties listed in Table 1.

Serological studies of IBV strains or isolates have shown that they may be grouped (5, 8, 13) but that these groups are not distinct serotypes and exhibit many interrelationships (5, 13). The results obtained in the present study in which all viruses of the Massachusetts serotype had similar polypeptide profiles and those differences that were detected between strains were limited to differences

in two glycopolypeptides do not exclude the possibility that all strains of IBV may share common antigens which may account for the complex serological relationships seen between the strains.

Addendum

During the preparation of this paper the authors received a preprint of a paper by Nagy and Lomniczi [Polypeptide patterns of infectious bronchitis virus serotypes fall into two categories. Arch. Virol. *61*, 341—345 (1979)] which was kindly supplied by Dr. B. Lomniczi, Veterinary Medical Research Institute of the Hungarian Academy of Sciences, Budapest, Hungary. In this paper the authors describe six major polypeptides of IBV with molecular weights: 75,000, 50,000, 45,000, 35,000, 28,000 or 24,000 and 22,000 which would appear to correspond to polypeptides p75, p54, p47, p40, p31 or p27 and p20—p23 of the present study. Nagy and Lomniczi further report that the strains of IBV they examined could be divided into two groups consisting of those viruses with a 28,000 molecular weight polypeptide: M41, Beaudette, PV, Lerida (all of Massachusetts serotype) and SE17, and those with a 24,000 molecular weight polypeptide: Connecticut (three strains tested), Gray, Iowa 97, Iowa 609 and T.

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