INFORMATION TO USERS

This dissertation was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

University Microfilms
300 North Zeeb Road
Ann Arbor, Michigan 48106
A Xerox Education Company
HUGHES, John Henry, 1942-

STUDIES ON THE ACID LABILITY OF RHINOVIRUSES.

The Ohio State University, Ph.D., 1972
Microbiology

University Microfilms, A XEROX Company, Ann Arbor, Michigan
STUDIES ON THE ACID LABILITY
OF RHINOVIRUSES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by

John Henry Hughes, B.S., M.A.

The Ohio State University
1972

Approved by

Donald C. Thomas
Adviser
Department of Medical Microbiology
PLEASE NOTE:

Some pages may have
indistinct print.
Filmed as received.

University Microfilms, A Xerox Education Company
ACKNOWLEDGMENTS

I wish to thank Dr. H. G. Cramblett, Chairman and Professor, Department of Medical Microbiology, and all other faculty members of this Department for providing a favorable environment that was very conducive to graduate learning and research.

I am especially indebted to Dr. D. C. Thomas, my adviser, for his time, patience, advice, and never ending efforts throughout all phases of this work.

A special note of thanks goes to my Graduate Committee members, Drs. V. V. Hamparian, N. L. Somerson, B. U. Bowman, and D. A. Wolff, for their excellent guidance and suggestions during the course of this investigation. I am particularly appreciative to the former two committee members for their personal encouragement and advice.

Gary VanWinkle, Judy Gebhart, Sharon Leach, Janet Moser, and Judy Halstead provided important technical assistance. I am very grateful to them for this.

Finally, my most sincere appreciation, thanks, and gratitude goes to my wife, Laura Jo, whose continued efforts, constant support, and encouragement contributed in an immeasurable way to the conclusion of this study.

ii
VITA

January 7, 1942. Born - Cleveland, Ohio

1964. B. S., The Ohio State University, Columbus, Ohio


1965-1967. Teaching Assistant, Department of Biology, Bowling Green State University, Bowling Green, Ohio

1967. M. A., Bowling Green State University, Bowling Green, Ohio

1967-1968. Teaching Assistant, Department of Zoology, University of Illinois, Urbana, Illinois

1968-1969. National Science Foundation Trainee, Department of Biology, Bowling Green State University, Bowling Green, Ohio

1969-1970. Research Associate, Department of Medical Microbiology, The Ohio State University, Columbus, Ohio

1970-1972. National Institute of Health Trainee, Department of Medical Microbiology, The Ohio State University, Columbus, Ohio


FIELD OF STUDY

Major field: Medical Microbiology

Studies of Rhinoviruses: Professor Donald C. Thomas
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>11</td>
</tr>
<tr>
<td>VITA</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>4</td>
</tr>
<tr>
<td>Historical Background of Rhinoviruses</td>
<td>4</td>
</tr>
<tr>
<td>Chemical and Physical Properties of Rhinoviruses</td>
<td>6</td>
</tr>
<tr>
<td>Biological Properties of Rhinoviruses</td>
<td>14</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>19</td>
</tr>
<tr>
<td>Cell Cultures</td>
<td>19</td>
</tr>
<tr>
<td>Viruses</td>
<td>21</td>
</tr>
<tr>
<td>Virus Purification</td>
<td>21</td>
</tr>
<tr>
<td>Viral Purity Test</td>
<td>23</td>
</tr>
<tr>
<td>Viral Assay</td>
<td>24</td>
</tr>
<tr>
<td>Acid Inactivation of Virus</td>
<td>25</td>
</tr>
<tr>
<td>Gradients</td>
<td>26</td>
</tr>
<tr>
<td>Precipitation of Macromolecules</td>
<td>29</td>
</tr>
<tr>
<td>Radioactivity Determinations</td>
<td>29</td>
</tr>
<tr>
<td>Polyacrylamide Gel Electrophoresis</td>
<td>30</td>
</tr>
<tr>
<td>RESULTS</td>
<td>34</td>
</tr>
<tr>
<td>Effect of Various Buffers on Two Human Picornaviruses</td>
<td>34</td>
</tr>
<tr>
<td>Effect of CsCl on Two Human Rhinoviruses</td>
<td>34</td>
</tr>
<tr>
<td>Effect of pH on the Inactivation of Human Rhinovirus Type 14</td>
<td>40</td>
</tr>
<tr>
<td>Effect of Time on the Inactivation of Human Rhinovirus Type 14</td>
<td>4.5</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Effect of Temperature on the Inactivation of Human Rhinovirus Type 14.</td>
<td>48</td>
</tr>
<tr>
<td>Effect of Acid Treatment on Recovery of Human Rhinovirus Type 14. Protein and Nucleic Acid.</td>
<td>48</td>
</tr>
<tr>
<td>CsCl and Sucrose Gradient Analysis of Acid Treated and Non-treated Radioactive Rhinovirus Type 14.</td>
<td>53</td>
</tr>
<tr>
<td>Polyacrylamide Gel Electrophoresis of Acid Treated and Non-treated Human Rhinovirus Type 14.</td>
<td>69</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>84</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>93</td>
</tr>
<tr>
<td>APPENDIX A (Media)</td>
<td>95</td>
</tr>
<tr>
<td>APPENDIX B (Viral Diluents)</td>
<td>99</td>
</tr>
<tr>
<td>APPENDIX C (Isotopes)</td>
<td>100</td>
</tr>
<tr>
<td>APPENDIX D (Buffers)</td>
<td>101</td>
</tr>
<tr>
<td>APPENDIX E (Polyacrylamide Gel Electrophoresis Materials)</td>
<td>103</td>
</tr>
<tr>
<td>APPENDIX F (Estimation of Molecular Weights by Internal Standards)</td>
<td>106</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>111</td>
</tr>
<tr>
<td>TABLE</td>
<td>Effect of Buffers on Two Human Picornaviruses</td>
</tr>
<tr>
<td>-------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>Effect of CsCl on Infectivity of Human Rhinovirus Type 14 After Storage at -22°C</td>
</tr>
<tr>
<td>2</td>
<td>Effect of CsCl on Infectivity of Two Rhinoviruses After Either Centrifugation or Storage at 0°C</td>
</tr>
<tr>
<td>3</td>
<td>Loss of Infectivity of Human Rhinovirus Type 14 at pH 3.0</td>
</tr>
<tr>
<td>4</td>
<td>Loss of Infectivity of Human Rhinovirus Type 14 After Lowering the pH from 5.0 to 3.0</td>
</tr>
<tr>
<td>5</td>
<td>Effect of Temperature on Infectivity of Human Rhinovirus Type 14 at pH 5.0</td>
</tr>
<tr>
<td>6</td>
<td>Effect of Acid Treatment on Human Rhinovirus Type 14 Protein</td>
</tr>
<tr>
<td>7</td>
<td>Release of RNA from Human Rhinovirus Type 14 After Acid Treatment</td>
</tr>
<tr>
<td>8</td>
<td>Molecular Weights of Poliovirus and Rhinovirus Polypeptides</td>
</tr>
<tr>
<td>9</td>
<td>Density Determination of Human Rhinovirus Type 14 Empty Capsids</td>
</tr>
<tr>
<td>10</td>
<td>Estimation of Molecular Weights by Internal Standards</td>
</tr>
<tr>
<td>FIGURE</td>
<td>DESCRIPTION</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>Inactivation of Purified Human Rhinovirus Type 1</td>
</tr>
<tr>
<td>2</td>
<td>Inactivation of Unpurified Human Rhinovirus Type 1</td>
</tr>
<tr>
<td>3</td>
<td>Inactivation of Human Rhinovirus Type 1</td>
</tr>
<tr>
<td>4</td>
<td>Radioactivity and Infectivity Profiles of Human Rhinovirus Type 1</td>
</tr>
<tr>
<td>5</td>
<td>Sucrose-Sedimentation Profile of Acid and 2-Mercaptoethanol Treated Tritiated Human Rhinovirus Type 1</td>
</tr>
<tr>
<td>6</td>
<td>Sucrose-Sedimentation Profile of Acid-Treated Tritiated Human Rhinovirus Type 1</td>
</tr>
<tr>
<td>7</td>
<td>Sucrose-Sedimentation Profile of Acid-Treated Tritiated Human Rhinovirus Type 1</td>
</tr>
<tr>
<td>8</td>
<td>Density Gradient Centrifugation of Acid-Treated Human Rhinovirus Type 1</td>
</tr>
<tr>
<td>9</td>
<td>Density Gradient Centrifugation of Acid-Treated Human Rhinovirus Type 1</td>
</tr>
<tr>
<td>10</td>
<td>Electrophoretic Profile of $^{14}$C-Labeled Poliovirus Type 2</td>
</tr>
<tr>
<td>FIGURE</td>
<td>PAGE</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>11</td>
<td>74</td>
</tr>
<tr>
<td>12</td>
<td>77</td>
</tr>
<tr>
<td>13</td>
<td>80</td>
</tr>
<tr>
<td>14</td>
<td>82</td>
</tr>
</tbody>
</table>
INTRODUCTION

Picornaviruses are small, single-stranded ribonucleic acid-containing viruses. The human picornavirus group is classified into the enterovirus and rhinovirus subgroups. Foot-and-mouth disease viruses (FMDV), which are classified as animal picornaviruses, and rhinoviruses (RV) are differentiated from other picornaviruses by acid lability in the absence of halide ions, and greater buoyant densities in cesium chloride (CsCl). The reason(s) for the greater density of FMDV and rhinoviruses has not been completely resolved. It has been proposed (71) that the increase in buoyant density of rhinoviruses may be due to a difference in hydration of the viral particle or to an increased binding of cesium ions. The reason(s) for the acid lability of FMDV and rhinoviruses is not known.

The enteroviruses: poliovirus, echovirus, and coxsackievirus, are acid stable. The cardioviruses: Maus-Elberfeld virus (ME), encephalomyocarditis virus (EMC), and Mengo virus (30), which are also grouped as animal picornaviruses and are chemically and physically indistinguishable from enteroviruses, can be either acid labile or stable depending on environmental conditions. At
pH 5.0 to 7.0 in the presence of 0.1 M chloride or bromide ions, Maus-Elberfeld virus is rapidly inactivated. This inactivation is dependent not only upon the presence of specific halide ions, but also on temperature and ionic concentration (89). Mengo virus inactivation behaves like that of ME-virus.

For rhinoviruses, Ketler et al. (56) reported the loss of infectivity after attempts to concentrate these viruses by precipitation at low pH. The loss of infectivity following acid treatment of rhinoviruses has since been reported by others (23, 24). The reason(s) for this extreme sensitivity of the rhinovirion to acid conditions has not been resolved. Little information is available on the mechanism(s), kinetics, or other events leading to this loss of infectivity following acid treatment of the rhinovirion. The only recent physico-chemical observation on the acid lability of rhinoviruses has been by Medappa et al. and Korant et al. (74, 59). Medappa and co-workers found that after acid treatment at pH 4.0 most of the viral proteins had aggregated. Korant and associates found a loss of rhinovirus polypeptide 4 (VP 4) and the production of "A" and "B" type particles following acid treatment of rhinoviruses at pH 5.0. The "A" particles resembled a slow natural component of rhinoviruses which they had previously isolated, while the "B" particles were analogous to "C" type particles of the poliovirus system.
Because of the limited amount of information available concerning acid lability of rhinoviruses, experiments were designed and undertaken to obtain more information on the effects of acid treatment on rhinoviruses. Inactivation as a function of time and pH was examined, as well as the effect of low pH on rhinovirus proteins and nucleic acid. The fate of radioactive rhinovirus proteins after acid treatment was followed by: (1) trichloroacetic acid precipitation, (2) sucrose and cesium-chloride gradient analysis, and (3) polyacrylamide gel electrophoresis (PAGE) analysis.
LITERATURE REVIEW

Historical Background of Rhinoviruses

The isolation of rhinoviruses from humans with common colds and the results of subsequent inoculation of human volunteers with these agents leaves little doubt of their etiological association with the common cold (11, 50, 102, 103, 106, 107, 108). The clinical signs and symptoms produced by rhinoviruses cannot be differentiated from those produced by other viruses causing upper respiratory tract illnesses. With rhinovirus infections, a coryzal syndrome is generally produced which is characterized by nasal congestion, sneezing, rhinorrhea, and a mild pharyngitis.

The rhinoviruses are classified as picornaviruses (75), but are differentiated from the other human picornavirus subgroup, the enteroviruses, on the basis of their density and acid sensitivity. As their name implies, they can be readily isolated from the nose and upper respiratory tract of individuals suffering from the common cold, but not from the gastrointestinal tract. In contrast, the enteroviruses are transient inhabitants of the gastrointestinal tract and are readily isolated from the inteste
tinal tract of man. Intestinal infection of man with rhinoviruses by enteric-coated capsules or by direct intestinal implantation has failed (12, 27, 68).

In 1953, a common cold virus was isolated by Andrewes and co-workers in England (1). This isolate was serially passed ten times in cell cultures, and colds were produced in volunteers with tenth cell passage material. Attempts to repeat this work were unsuccessful and attributed to differences in cell cultures used to pass the virus. In 1956, two independent American laboratories simultaneously isolated the first rhinoviruses from patients with common cold-like upper respiratory tract illnesses (81, 85). The two American isolates were designated GL 2060 and J. I. These viruses were found to be closely related and were initially classified as echovirus 28. Today they are recognized as human rhinovirus type 1A (HRV-1A).

In the early 1960's, the viruses isolated by Tyrrell and co-workers in Salisbury, England, from patients suffering with colds, were named Salisbury viruses (110). In 1961, Hamparian et al. (37) using diploid human fetal lung fibroblastic cell cultures, isolated several distinct viruses from humans. Since the latter isolates were immunologically distinct from the Salisbury viruses, they were called coryzavirus (37). In 1961, Andrewes formally proposed that viruses isolated from humans with colds be
called rhinoviruses (2). This name has since been adopted (109).

Early investigations revealed many serotypes of rhinoviruses. Seven distinct serotypes were isolated by Taylor-Robinson and Tyrrell (104), 20 by Hamparian et al. (37, 38), four by Johnson and Rosen (53), and 20 by Connelly and Hamre (20, 41). To facilitate the comparison of rhinovirus strains isolated by different investigators, an official international numbering system for the first 55 rhinovirus serotypes was adopted (15). A distinct rhinovirus serotype was defined as one which was not neutralized by 20 antibody units of antisera to any other serotype and whose antiserum did not neutralize three to 320 tissue culture infectious dose, 50 per cent (TCID_{50}) of any other rhinovirus type. Since the collaborative report of 1967 (15), the number of distinct rhinovirus serotypes has been increased officially to 89 (16). More serotypes are expected to be identified.

**Chemical and Physical Properties of Rhinoviruses**

**Size, shape, and density.** Early electron microscopic observation, ultrafiltration studies, and sedimentation analysis revealed that rhinoviruses were small in size, 17 to 18 nanometers (nm) in diameter (37). Other ultrafiltration studies of rhinoviruses revealed that these viruses were less than 50 nm in size (24). Recent
electron microscopy has shown rhinoviruses to possess isosahedral symmetry and to be 15 to 30 nm in diameter (33, 70, 71, 73). Thirty-two capsomers have been reported for rhinoviruses and other picornaviruses (70), but the possibility of the existence of 42 or 60 capsomers has not been completely ruled out (89).

The density of rhinoviruses in solutions of cesium chloride, a salt of a heavy cation, is from 1.38 to 1.42 g/cc (13, 22, 23, 32, 33, 51, 57, 58, 59, 73, 74, 105). In salts of heavy anions, such as potassium citrate or potassium tartrate, rhinoviruses are not as dense. The reason(s) for this density effect in cesium is not known. When rhinoviruses were acid treated at pH 5.0, an artificial dense component was observed in cesium-chloride gradients (59). This artificial dense component had a density of 1.45 g/cc. If rhinovirus samples were brought back to neutral pH after acid treatment at pH 5.0, components having densities of 1.29 g/cc and 1.31 to 1.40 g/cc were observed (59).

Nucleic acid and protein. Rhinoviruses replicate in the presence of 5-fluoro-2'-deoxyuridine which is a halogen substituted pyrimidine analogue that inhibits the replication of deoxyribonucleic acid (DNA) viruses (56). Infectious ribonucleic acid (RNA) has been recovered from rhinoviruses and has been shown to be susceptible to RNase, but not DNase (56, 94). Base analysis of rhinovirus RNA has
shown that uridylic and adenylic acids are not present in a one-to-one ratio (72); therefore, the RNA of rhinoviruses is single stranded. The proportion of RNA in rhinoviruses is approximately 30 per cent of the total viral mass (72). Foot-and-mouth disease virus, EMC-virus, and poliovirus all possess similar amounts of RNA (3, 4, 91). By measuring the contour-strand lengths of ribonucleoprotein (RNP) isolated from rhinovirus type 1B after heating, McGregor and Mayor (71) estimated the molecular weight of rhinovirus RNA to be about 4 x 10^6 daltons. Chemical and hydrodynamic analysis of the rhinovirion gave a molecular weight of 2.1 x 10^6 daltons for the RNA (72). Since the modal length of the RNP may not be a real measure of the RNA content, the higher value has not been accepted as the true molecular weight of the rhinovirion RNA. Other estimates of rhinovirus RNA molecular weight have been 2.2 x 10^6 daltons (94) and 2.4 to 2.8 x 10^6 daltons (8). Foot-and-mouth disease virus has an RNA molecular weight of about 2.8 x 10^6 daltons (115); poliovirus, 2.6 x 10^6 daltons (34, 102); and EMC, 2.7 x 10^6 daltons (9). Thus, the molecular weight of rhinovirus RNA is in the range of other picornaviruses.

After treatment with dimethylsulfoxide (DMSO), rhinovirus and poliovirus RNA were found to have molecular weights of very similar size (78). Treatment with DMSO destroys the secondary structure of RNA so that the
distance traveled by each RNA species in a gradient is considered to be a reflection of size, and not structure (100).

The sedimentation coefficient for rhinovirus RNA extracted from purified virions is about 32S (8, 94). The RNA extracted from rhinovirus-infected cells has the same sedimentation coefficient (94). An equine rhinovirus, foot-and-mouth disease virus, and a porcine enterovirus have been found to have RNAs of similar sedimentation coefficients (8). When rhinovirus, porcine enterovirus, and foot-and-mouth disease virus RNAs were treated with 6.0 per cent formaldehyde, to eliminate configurational differences (31), all had similar sedimentation coefficients (8). These results suggest that the RNA of all these viruses are similar in size.

It is not known what effect acid treatment has on the nucleic acid of rhinoviruses. Mengo virus RNA appeared to maintain its integrity following acid treatment at pH 6.2 (65). Infectious RNA has been recovered from foot-and-mouth disease virus after acid inactivation at pH 5.6 or 6.5 (7, 77).

In infected cells, rhinoviruses induce not only 32S RNA molecules, but also an 18S RNA species that is partially resistant to degradation by ribonuclease (94). The 18S component may be a mixture of double-stranded replicative forms (RF) and replicative intermediates (RI) which
has been observed in poliovirus-infected cells (4, 5, 84, 114).

Rhinoviruses consist of approximately 70 per cent protein. The partial specific volume ($\bar{v}$) of these proteins calculated from amino acid composition is $0.738 \text{ cm}^3/\text{g}$ (72). The partial specific volume of the complete rhinovirus virion is $0.682 \text{ cm}^3/\text{g}$ (72). The molecular weight of the complete rhinovirus virion calculated from the Svedberg equation is $7.1 \times 10^6$ daltons (72). Therefore, the protein components of rhinoviruses weigh approximately $5.0 \times 10^6$ daltons.

Four polypeptides (VP 1, 2, 3, and 4) are liberated following sodium dodecyl sulfate (SDS) treatment of rhinoviruses (48, 57, 59, 74). Poliovirus, Maus-Elberfeld virus, echovirus, bovine enterovirus, and M-mengo virus all have been shown to have four structural polypeptides associated with the infectious virus particle (54, 57, 79, 88, 101). Controversy still exists as to the number of polypeptides in foot-and-mouth disease virus (10, 60, 112, 113, 116). However, using procedures similar to those used for other picornaviruses, four polypeptides have been observed for FMDV (10). For rhinoviruses, the four polypeptides appear in equimolar proportions (74).

Korant et al. (59) found three polypeptides present in rhinovirus empty capsids (VP 0, 1, and 3). A dense component observed, by the same investigators, following
CsCl gradient purification was 0.04 to 0.05 g/cc greater in density than mature virions. An artificial dense component, produced by acid treatment at pH 5.0, was found to contain VP 1 and VP 3. Rhinovirus "A" and "B" type particles induced after pH 5.0 treatment were found to lack VP 4 (59).

Acid lability. In 1960, Tyrrell and co-workers showed that the ability of a rhinovirus to cause colds, produce interference, or a cytopathic effect in cell cultures could be abolished by overnight treatment at pH 2.0 (108). In 1962, Ketler et al. (56) demonstrated a significant loss of infectivity following incubation of common cold viruses at pH 3.0 for three to four hours at room temperature. Since these initial observations on the acid lability of rhinoviruses, only two reports have appeared, all within the past year, concerning the physicochemical events following the acid inactivation of rhinoviruses (59, 73). After pH 4.0 treatment of rhinovirus type 1A for either 10 or 60 minutes and subsequent sedimentation analysis on 10 to 30 per cent glycerol gradients, greater than 80 per cent of the viral material sedimented to the bottom of the gradient. Only six to 14 per cent of the viral material was not pelleted (74).

Korant et al. (59) observed upon cesium-chloride density gradient analysis of pH 5.0 treated virus, a component similar to a previously identified "dense component". This acid-induced component resembling the
"dense component" was found to contain VP 1 and VP 3. Sucrose gradient analysis, of acid-treated (pH 5.0) and then neutralized samples, revealed two peaks designated as "A" and "B" particles. "A" and "B" particles were found to have S values of 135S and 75S respectively. The "A" particles contained RNA and resembled the slow natural component that was isolated from crude viral preparations. Most of the slow natural component banded, like a dense component at a density of 1.45 g/cc. The density of the acid-induced "A" particles was found to be variable (1.31 to 1.40 g/cc) and did not give rise to a dense component. The "B" particles were found to have a density of 1.29 g/cc which is analogous to that of empty capsids. The poly­peptide compositions of the acid-induced "A" and "B" particles were the same, that is both lack VP 4. Treatment of an equine rhinovirus under similar conditions did not result in the induction of "A" and "B" type particles.

After sucrose gradient centrifugation of pH 6.5 treated radioactive FMDV, Burroughs et al. (10) found a 12S peak that had approximately 80 per cent of the initial radioactivity. The remainder of the radioactivity was at the bottom of the gradient. SDS-polyacrylamide gel electrophoresis of the 12S component revealed the presence of the three major polypeptides. The fast-migrating polypeptide of the intact virus was not present with the 12S component. In contrast, SDS-polyacrylamide gel electrophoresis of the
pelleted viral material revealed the presence of the fast-migrating component as well as trace amounts of the other three major polypeptides. Fractionation of acid-treated FMDV on carboxylic acid resin resulted in the nucleic acid and fast-migrating component passing through the column. The other three major polypeptides were selectively absorbed and eluted as a peak.

The only other picornaviruses inactivated by low pH are the cardioviruses. Conditions for acid inactivation of these viruses are specific and limited. For example, Mak et al. (65) found that maximum inactivation of Mongo virus variants occurred at pH 6.2 in the presence of 0.1 to 0.2 M chloride or bromide ions. High tonicity of these ions, 0.5 M or greater, resulted in little loss of infectivity at pH 6.2. No inactivation occurred in the presence of fluoride or iodide ions. Finally, at pH 4.0 these viruses were stable in the absence of chloride or bromide ions.

The inactivation of Mongo virus variants under the appropriate acid conditions results in the formation of three components: a 13 to 15S component, material that is pelleted, and a top component. The 13 to 15S component was found to contain VP 1 and VP 2. Pellets and top components contained essentially VP 1, 2, and 3 (65, 66, 80).

Acid inactivation of another cardiovirus, ME-virus, is similar to that of Mongo viruses. Dunker and Rueckert (29) found acid treatment of ME-virus yielded a 14S compo-
ment and viral material that was pelleted. The pellets contained all of the viral polypeptides while the 14S material contained three structural polypeptides.

**Biological Properties of Rhinoviruses**

**Site of replication.** Hamparian *et al.* (37) observed, by electron microscopy, rhinoviruses in the cytoplasm of infected cells. The presence of rhinoviruses in the cytoplasm of infected cells has since been confirmed by Blough *et al.* (6) and by Kwana and Matsumoto (55). The latter workers demonstrated, by electron microscopy time-sequence studies, the assembly of rhinoviruses in the cytoplasm of infected cells. Indirect-fluorescent-antibody studies have also been used to show the appearance of rhinoviruses in the cytoplasm of infected cells (28). A strong relationship existed between the appearance of immunofluorescence in the cytoplasm and intracellular virus as determined by infectivity.

**Host range and growth patterns of rhinoviruses.** Rhinoviruses can be isolated from humans year around with infections being more common in cooler seasons (36, 42). Rhinoviremia is rare or practically non-existant (25, 26); only two cases have ever been reported of recovery of rhinoviruses from the blood (111). There is no evidence that rhinovirus infection changes the bacterial flora in a quantitative or qualitative manner. Attempts to produce
rhinovirus infection in frogs, guinea pigs, gerbils, marmosets, mice, ferrets, and hamsters have all failed (83). To date, only the gibbon has been repeatedly susceptible to rhinovirus infection (83).

Some human rhinoviruses were initially found to replicate only in human kidney cells, while others would replicate in monkey and human kidney cells (107). The latter were designated M strains, the former H strains. Rhinoviruses have since been adapted to such heteroploid cell lines as HEp-2, KB, and HeLa cells (45, 52, 56, 76, 96). In 1969, Stott and Heath (98) reported the growth of rhinovirus type 2 in suspended cultures of L 132 cells. Thomas et al. (105), a year later, described the growth of rhinovirus type 14 in replicating HeLa cell suspension cultures. Such cultures were used to obtain large amounts of virus and to conduct precise studies on rhinovirus replication. The isolation of rhinoviruses that fail to grow in some cell culture systems and only replicate in organ cultures is rare (47).

Complement fixation (CF), hemagglutination (HA), immunodiffusion (ID), and neutralizing activity. Because of low titered rhinovirus preparations, complement fixation tests have not been routinely used. CF activity is associated with both non-infectious (C) and infectious (D) particles. Following isopycnic centrifugation of rhinovirus type 1A, Dans et al. (22) found 16 to 30 per cent and
60 to 75 per cent of the CF activity associated with the infectivity peak (density 1.41 g/cc) and empty capsid peak (density 1.30 g/cc) respectively. Heterotypic hyperimmune antiserum of rhinovirus type 1A was found to participate in CF activity only with the less dense CF antigen of rhinovirus type 2. Thus the dense and less dense antigens have different specificities; the former, type specific and the latter, group specific. Serologic differences in specificity between dense and less dense viral antigens have been reported by Hummeler and Hamparian (49) for polioviruses, and by Schmidt and co-workers (92) for coxsackieviruses. The effect of acid treatment of rhinoviruses on CF activity is not known.

Recently, rhinoviruses have been shown to hemagglutinate guinea pig, hamster, dog, sheep, and rabbit erythrocytes (99). Erythrocytes from chick, goose, horse, rhesus monkey, African green monkey, and human group-O were not agglutinated by rhinoviruses. Hemagglutination was found to be pH and temperature dependent, with titers highest at pH 8.0 and 9.0, and hemagglutination occurring at room temperature and 4°C. Hemagglutination was inhibited when sheep erythrocytes were treated with filtrates of Vibrio cholerae, but not when treated with trypsin. Thus the receptor appears to be sensitive to neuraminidase, and the hemagglutinin may be similar to that of cardioviruses rather than other picornaviruses. After
viral samples were exposed to pH 4.0, 8 M urea, or heat, hemagglutination activity was abolished. In sucrose and cesium-chloride gradients, the hemagglutinin was associated with the viral particle. Finally, antisera inhibited hemagglutination and convalescent antisera contained increased amounts of hemagglutinating inhibiting antibody.

Human sera, with specific neutralizing antibody activity, give two-precipitin lines against the homologous rhinovirus antigen in an immunodiffusion (ID) test (14). Sera lacking specific rhinovirus neutralizing antibody form one precipitin line when reacted with specific rhinovirus antigen (14). Thus, rhinoviruses have a common group antigen as well as strain-specific antigens as revealed by the ID test. In ID tests, human rhinoviruses were also found to share a group antigen with other human picornaviruses (14). Acid-treated rhinovirus antigens or antiserum prepared to acid-treated antigens have not been used in ID tests.

The neutralizing antibody response to rhinoviruses has been shown to be highly type specific (15, 39). Hamparian et al. (39) found that heterotypic responses following infection with rhinoviruses were rare. Phillips and co-workers (82) found a homotypic antibody response following rhinovirus infection in students. One particular student had five different rhinovirus infections within a year without demonstrable heterotypic responses. In the
past, the hallmark for classifying rhinovirus serotypes has been by the neutralization test (18). Preliminary experiments in our laboratory have revealed that acid treatment of rhinoviruses abolishes the ability to elicit a neutralizing antibody response (40).
MATERIALS AND METHODS

Cell Cultures

Viruses were propagated in HeLa cells originally obtained from Microbiological Associates, Bethesda, Maryland. Cells were cultivated in monolayers and in suspension cultures. Monolayer cultures were grown in three different size tissue culture containers. These tissue culture containers were: (1) 32 ounce Brockway prescription bottles, (2) three ounce Brockway prescription bottles, and (3) 16 x 22 mm tissue culture tubes. Brockway prescription bottles were obtained from the Ohio Container Company, Columbus, Ohio while the tissue culture tubes were obtained from Bellco Glass, Inc., Vineland, New Jersey. Suspension cultures were grown in 50 to 2,000 ml side arm spinner flasks (Bellco Glass, Inc.). Cell cultures were held in suspension by using magnetic stir bars and a Forma induction magnetic drive incubator (Forma Scientific, Marietta, Ohio). Before initial use, spinner flasks were treated with silicone (Clay-Adams, Inc., New York).

For cell passages, maintenance medium (Appendix A) was removed from 32 ounce prescription bottles and 8.0 ml of 0.25 per cent trypsin solution (Difco Laboratories,
Detroit, Michigan) was added to the cell monolayers. After one to two minutes, the trypsin was decanted and the bottles were incubated at $37^\circ$ C for five to ten minutes or until the cells detached from the glass. After detachment, cells were suspended in 10 ml of growth medium (Appendix A). Viable cell counts were determined by making a 1:2 dilution of cells in 0.2 per cent trypan blue and counting cells that excluded dye in a hemocytometer under 100X magnification.

Thirty-two ounce prescription bottles were seeded with enough cells, approximately two to four million in 75 ml of growth medium, to form confluent cell monolayers in three to five days. Three ounce prescription bottles were seeded: (1) with three million cells in 10 ml of growth medium for use within one day, (2) with one million cells in 10 ml of growth medium for use within two to three days, or (3) with one-half to three-quarter million cells in 10 ml of growth medium for use within four days. HeLa cell tubes were prepared routinely twice a week. The tubes were seeded with either 175,000 cells or 125,000 in 1.0 ml. On alternate two and four day intervals the growth medium was removed from the tubes and replaced with 1.5 ml of maintenance medium.

Suspension cultures were initiated in 50 ml of spinner medium (Appendix A) containing approximately $4 \times 10^7$ cells. Fresh spinner medium was added as cell
concentrations increased. Cultures were routinely carried in 500 ml of medium in a one liter flask with a concentration of 3.0 to 6.0 x 10^5 cells/ml. Cells were counted daily and were maintained at the above concentration by discarding a portion of the culture and adding fresh spinner medium. Viable cell counts were done as described above, but 0.2 per cent eosin-Y stain was used instead of trypan blue.

Viruses

Human rhinoviruses type 14 (HRV-14), strain 1059; type 20 (HRV-20), strain 15-CV19; and poliovirus type 2 Sabin were used. All were obtained from Dr. V. V. Hamparian, Departments of Medical Microbiology and Pediatrics, College of Medicine, The Ohio State University, Columbus, Ohio.

Virus Purification

Monolayer cultures were infected at a multiplicity of 0.1 to 1.0 plaque forming units (PFU) per cell. Virus was added directly onto cells that were drained of their tissue culture medium. Viruses were allowed to adsorb for two hours at 33° or 37° C. Following adsorption, 40 to 60 ml of maintenance media were added to the monolayers. Rhinovirus infected cultures were then incubated at 33° C on a rocking platform (Model 6700, Bellco Glass, Inc.) at three to six cycles per minute. Poliovirus infected cultures were handled similarly but were incubated at 37° C.
in a stationary position. When infection was 100 per cent complete (four plus cytopathic effect, CPE), cells were frozen (-20° C) and thawed three times. After the last freeze-thaw cycle the cell lysates were clarified by centrifugation at 400 g for ten minutes in a Model K International centrifuge. Two volumes of the clarified cell lysates were mixed with one volume of trichlorotrifluoroethane (Freon 113, E. I. DuPont de Nemours Company, Wilmington, Delaware) and blended at maximum speed for three one-minute intervals in a Sorvall omni-mixer (Ivan Sorvall, Inc., Norwalk, Connecticut), submerged in an ice bath. Following homogenization, the mixture was clarified by centrifugation at 900 g for ten minutes. After clarification, the viral supernatant was frozen at -30° C. Upon thawing, the supernatant was clarified again by centrifugation at 900 g for ten minutes. The virus was then concentrated by centrifugation in a No. 30 fixed-angle rotor at 30,000 RPM (105,651 g) for four hours at 5° C in a Model L Spinco preparative ultracentrifuge (Beckman Instruments, Inc., Palo Alto, California). The viral pellets were resuspended in either 0.01 M Tris or sodium-phosphate buffers (Appendix D) and subjected to cesium-chloride isopycnic centrifugation in an SW 39 rotor at 39,000 RPM (175,296 g) for 48 or 60 hours.

Suspension cultures were infected by a procedure similar to that described by Thomas et al. (105). The
cells were concentrated by centrifugation at 400 g for ten minutes and then resuspended in maintenance medium at one-tenth the initial volume. Virus was added and allowed to adsorb for two hours at 33°C. After adsorption, the cells were diluted to the original concentration by adding fresh maintenance medium. At the end of the viral growth period, the cells were concentrated again by centrifugation at 400 g for ten minutes, resuspended in 10 ml of maintenance medium, and frozen at -30°C. Thereafter, the virus was purified and concentrated as described above.

Viral Purity Test

Viral stocks were routinely tested for purity in the following manner. Rhinovirus type specific hyperimmune guinea pig serum, obtained from Dr. V. V. Hamparian, was heat inactivated at 56°C for 30 minutes. After heat inactivation, serum was diluted 1:10 in viral diluent (Appendix B) if the neutralizing antibody titer was less than 3,000 when tested against 30 to 300 TCID₅₀ of virus. (Neutralizing antibody titer is the reciprocal of the highest dilution of serum that completely inhibits the replication of the input virus.) If the serum titer was greater than 3,000 it was diluted 1:20 and serum with a titer of less than 100 was used at a dilution of 1:4.

Four parts of diluted serum (2.0 ml) were mixed with one part (0.5 ml) of undiluted virus having a titer of
approximately $5 \times 10^6$ PFU/ml. The viral-serum mixture was incubated for two hours at room temperature. After the first hour, the viral-serum mixture was transferred to a fresh container. After two hours, the total viral-serum mixture (2.5 ml) was distributed evenly into ten HeLa tube cultures. These cultures were examined daily for five days for evidence of CPE. A purity test was considered satisfactory if no CPE was detected after five days. In order to determine the viral input of virus used in a purity test, virus was titrated simultaneously by the TCID$_{50}$ method.

**Viral Assay**

**Plaque titrations.** Viruses were plaqued by the method reported by Conant et al. (19). Briefly, three ounce prescription bottle cultures of HeLa cells, having nearly confluent monolayers, were infected by adding 0.5 ml of the appropriate viral dilutions onto cells that were previously drained of their tissue culture medium. Two bottles were used for each dilution. Following viral adsorption (two hours at 33°C), the excess inoculum was poured off and 5.0 ml of the initial overlay medium (Appendix A) were added to each bottle. When the initial overlay medium had solidified, the bottles were inverted and incubated at 33°C. After four to five days, 7.0 ml of the final overlay medium containing 0.007 per cent neutral red (Appendix A) were added to each bottle. After
solidification, the bottles were again inverted and incubated at 33°C in the dark by covering the bottles with aluminum foil. The following day, plaques were counted and the titers were determined with the following formula:

\[
\text{PFU/ml} = \frac{T}{(I)(D)}
\]

where \(T\) equals total number of plaques per dilution, \(D\) equals dilution, and \(I\) equals inoculum volume in milliliters.

**TCID\text{50} titrations.** Two to five HeLa tube cultures were each inoculated with a 0.1 ml of a viral dilution. Rhinovirus titrations were incubated at 33°C on roller drums and poliovirus titrations were incubated at 37°C in a stationary position. Tube cultures were examined daily for five days for cytopathic effects (CPE). Infectivity titers were determined by the method of Reed and Muench (86).

**Acid Inactivation of Virus**

For polyacrylamide gel electrophoresis (PAGE) and gradient analysis, purified radioactive rhinovirus type 14 was subjected to pH 3.0 treatment by one of the following methods: (1) dialyzing against pH 3.0 citrate-phosphate buffer (Appendix D) for two hours at room temperature, (2) making a 1:2 dilution of virus in pH 3.0 citrate-phosphate buffer and incubating for 15 minutes at room temperature, or (3) by adding H HCl and incubating for two
hours at room temperature. After acid treatment, samples were used either directly, or were brought to pH 7.0 by adding $N$ NaOH, or by dialyzing against 0.01 M sodium-phosphate buffer. When specified, inactivation was carried out in the presence of 1.0 per cent 2-mercaptoethanol, 2-ME (Eastman Kodak Company, Rochester, New York).

Rhinovirus inactivation studies as a function of pH were carried out in the following manner. Viral suspensions containing $2 \times 10^7$ to $2 \times 10^8$ PFU/ml were diluted 1:10 (final volume 2.0 ml) in citrate-phosphate buffers ranging in pH from 3.0 to 8.0. After two hours incubation at room temperature, the amount of infectious virus remaining was determined by plaque or tube assay. For kinetic studies, 0.2 ml of rhinovirus type 14 at an approximate concentration of $5 \times 10^6$ to $6 \times 10^7$ PFU/ml was incubated with 0.8 ml of pH 5.0 citrate-phosphate buffer or 10X MEM (pH 3.0). At the desired time intervals, inactivation was stopped by adding 1.0 ml of pH 8.0 citrate-phosphate buffer. The amount of infectious virus remaining was determined by plaque assay. The pH of all buffers was determined by using an Orion digital pH meter, Model 801.

Gradients

Sucrose and cesium-chloride gradients were used to analyze radioactive rhinovirus type 14 that was acid treated at pH 3.0. Ribonuclease-free density gradient grade sucrose
(Schwarz/Mann, Orangeburg, New York) was used to make linear continuous 15 to 30 per cent sucrose gradients. Diluents for these gradients were either sodium-phosphate buffer pH 6.8 or reticulocyte standard buffer (RSB) pH 7.6 (Appendix D). An LKB refrigerated fraction collector (LKB, Stockholm, Sweden) was used to collect approximately 0.5 ml volume fractions from sucrose gradients. Fractions were collected by pumping the gradient contents (by means of a peristaltic pump) through a 20 gauge stainless steel cannula inserted from the top of the gradients to the bottom. Absorbancy at 280 nm for each sucrose fraction was either determined manually or monitored by a flow-through cell connected to a Gilford 240 Spectrophotometer equipped with a recorder (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). To insure linearity of sucrose gradients, a Bausch and Lomb Abbe-3L Refractometer (Bausch and Lomb, Rochester, New York) was used to measure the refractive index of the fractions collected. All measurements were carried out at room temperature. For estimation of sedimentation coefficient values in sucrose gradients, the procedure of Martin and Ames (67) was used. Purified rhinovirus protein having a density of 1.30 g/cc, purified infectious rhinovirus type 14, and purified infectious poliovirus type 2 were used as sedimentation markers. The sedimentation coefficient values for these components are approximately 75 to 80S and 150 to 160S (59, 72).
Cesium-chloride (CsCl) gradients were generated by centrifugation. CsCl powder was obtained from General Biochemicals, Chagrin Falls, Ohio. The amount of CsCl needed for 5.0 ml gradients was determined by using the following formula:

\[
\text{grams CsCl} = \frac{\text{gradient weight per}}{\text{mean density}} \times \frac{\text{volume}}{\text{mean of mean density}}
\]

The appropriate amounts of CsCl, diluent, and virus were added to 5.0 ml (one-half inch by two inches) Beckman cellulose nitrate centrifuge tubes (Spinco Division, Palo Alto, California). Diluent for CsCl gradients was either 0.01 M Tris buffer pH 8.1 or 0.01 M sodium-phosphate buffer pH 6.8. After mixing, the samples were centrifuged in an SW 39 rotor at 39,000 RPM (175,296 g) for 48 or 60 hours at 5°C in a Spinco L preparative ultracentrifuge. At the completion of isopycnic centrifugation runs, the bottoms of centrifuge tubes were punctured with a 22 gauge needle and fractions consisting of 25 drops were collected. Densities, infectivity, and/or radioactivity were determined on appropriate fractions. Cesium salt was removed by dialysis against a 2,000 ml volume of 0.01 M Tris or sodium-phosphate buffers at 4°C. Before use, dialysis tubing was boiled for ten minutes in 0.1 M EDTA, followed by several washes in hot distilled water. Treated tubing was stored at 4°C in distilled water.
Trichloroacetic Acid (TCA) Precipitation of Radioactive Macromolecules

Radioactive viral macromolecules collected from sucrose and CsCl gradients were assayed for radioactivity after TCA precipitation. Bac-T-Flex type B-6 filters, 25 mm (Carl Schleicher and Schuell Company, Keene, New Hampshire) were used to collect TCA precipitated macromolecules. Filters were pre-soaked at least one hour in distilled water and used wet.

Samples ranging in volume from five lambda to 500 lambda were added to 3.0 ml of cold 0.6 M TCA. Bovine serum albumin (Pentex Corporation, Kankakee, Illinois) at a concentration of 100 μg/0.1 ml was added as a carrier. The samples were mixed and held in an ice bath for one to four hours. After collection of precipitates, the membranes were washed with 12 ml of cold 0.3 M TCA, and air dried either overnight at room temperature or at 80° C for two hours. The amount of radioactivity on membranes was determined with a Packard liquid scintillation counter (Packard Instrument Company, Inc., Downers Grove, Illinois).

Radioactivity Determinations

Radioactivity was assayed in a liquid scintillation spectrometer (Model 3310-S) with bialkali photomultiplier tubes and automatic external standardization mode. Solvent and scintillation cocktail for acrylamide gels consisted of
0.1 per cent water, 10 per cent NCS, 0.60 per cent 2,5-diphenyl-oxazole (PPO), and 0.0075 per cent 1,4-bis 2-[5-Phenyborazolyl benzene], POPOP, (all from Amersham/Searle Corporation, Clearbrook, Illinois) in 15 ml of toluene (Fisher Scientific Company, T-324, Fair Lawn, New Jersey). Scintillation cocktail for TCA precipitated gradient fractions consisted of 0.105 per cent 2,5-bis[2-(5-tert-Butylbenzoxazolyl)]-Thiophene, BBOT, in (Packard Instrument Company) in toluene. Efficiencies were determined by using \(^3\text{H}\) and \(^{14}\text{C}\) toluene as a known radioactive source. Efficiencies for single labeled experiments were 41.5 per cent for \(^3\text{H}\) and 86.5 per cent for \(^{14}\text{C}\). Efficiencies for double labeled experiments were 23.2 and 54.2 per cent for \(^3\text{H}\) and \(^{14}\text{C}\), respectively, with the efficiency of \(^{14}\text{C}\) in the tritium channel being 5.1 per cent. Disintegrations per minute (DPM) were determined by dividing the counts per minute (CPM) by the efficiency. Quenching was found to be insignificant for the amount of protein sample used.

**Polyacrylamide Gel Electrophoresis (PAGE)**

The structural components of rhinovirus type 14 before and after treatment with acid were determined by PAGE. Reagents for polyacrylamide gel electrophoresis are listed in Appendix E. Stock solutions for the preparation of sodium dodecyl sulfate (SDS) gels are essentially the same.
as those reported by Maizel (64). Acrylamide was recrystallized from chloroform and bis acrylamide from acetone by a procedure similar to that reported by Loenig (61). Ten per cent gels, 80 mm or 210 mm in length, were allowed to polymerize in glass tubes 6 mm in diameter. To reduce gel bonding to glass and facilitate removal of gels, glass tubing was cleaned in 2.0 per cent NaOH and then treated with 1.0 per cent silicone (Clay Adams Siliclad). Detergent gels contained 10.0 per cent (w/v) acrylamide, 0.26 per cent (w/v) N, N'-bis-methylene acrylamide, 0.05 per cent (v/v) N, N, N', N'-tetramethylethlenendiamine, 0.10 per cent (w/v) ammonium persulfate, 0.1 per cent SDS, and 0.1 M sodium-phosphate buffer with distilled water as diluent. Buffer for detergent gels consisted of 0.1 per cent SDS and 0.1 M sodium-phosphate buffer. Five per cent non-detergent gels were similar to 10 per cent gels, but consisted of Tris base, EDTA, and boric acid as diluent and buffer (Appendix D).

Detergent gels were pre-electrophoresed for one hour at 6 mA/gel to remove any unpolymerized reactants. Non-detergent gels were pre-electrophoresed for one hour at 2 mA/gel. Yeast alcohol dehydrogenase, bovine trypsin (both from Miles Laboratories, Inc., Kankakee, Illinois), bovine serum albumin (Pentex Corporation, Kankakee, Illinois), and B. subtilis alpha amylase (Calbiochem, Elk Grove, Illinois) were used to calibrate SDS gels for
molecular weight determinations. SDS samples were prepared as reported by Medappa et al. (74). Non-SDS samples were inactivated at pH 3.0 with 2-ME as mentioned previously. Sample sizes from 100 to 200 μl in 10.0 per cent sucrose and having 5 μg of bromphenol blue tracking dye were layered on top of the gel column under the electrode buffer. Detergent samples were electrophoresed into the gel at 2 mA/gel for one hour after which the current was increased to 6 mA/gel for 18½ hours. Non-detergent samples were electrophoresed into the gel at 0.5 mA/gel for one hour and then increased to 1.0 mA/gel for 100 minutes. Following electrophoresis, gels were mechanically fractionated into scintillation vials by slicing into 1.0 mm slices (Gilson Medical Electronics Gel Fractionator, Middleton, Wisconsin). Gel slices were dried overnight at 50°C. After drying, 0.01 ml of distilled water and 1.0 ml of NCS tissue solubilizer (Amershan/Searle Corporation, Clearbrook, Illinois) were added. Solubilization was carried out for two hours at 50°C. After solubilization, the samples were cooled to room temperature, the appropriate scintillation cocktail added, and radioactivity determined in a liquid scintillation counter. Gels that were not sliced were fixed for 18 hours in 10.0 per cent TCA, stained for 18 to 24 hours in 0.25 per cent Coomassie brilliant blue R250, and destained with several changes of 7.0 per cent acetic acid at 45°C.
The migration of protein or polypeptide bands were expressed as direct mobility from the origin or as a relative mobility \( R_m \) which is defined as the ratio of distance (d) moved by a protein to the distance (d) moved by a marker (bromphenol blue or some marker protein),

\[ R_m = \frac{d_{\text{protein}}}{d_{\text{marker}}} \]

The logarithm of molecular weight when plotted against direct mobility or \( R_m \) results in an exponential relationship which can be expressed as:

\[ Y = b e^{-k} \quad (95) \]

where \( Y, b, e, \) and \(-k\) represent molecular weight, \( Y \) intercept, base of natural logarithm, and distance of migration of a protein respectively. The slope of the above equation was determined by fitting my empirical protein standard data with linear regression by the method of least squares. By using the above standard molecular weight plot, the molecular weight for each unknown viral component could be estimated. In addition, internal standards (poliovirus polypeptides 1 to 3) were used to estimate the molecular weights of unknown rhinovirus type 14 structural components. The Appendix contains a method of estimating molecular weights of unknown components by using internal standards.
RESULTS

Effect of Various Buffers on Two Human Picornaviruses

Before acid inactivation experiments on human rhinovirus type 14 were attempted, experiments were done to assure that the buffers were not viracidal. Poliovirus type 2 and rhinovirus type 14 were incubated for three hours at room temperature in the presence of 10X MEM (pH 3.0) or in citrate-sodium phosphate buffers (pH 3.0 and 8.0). The initial concentration of the citrate and phosphate was 0.1 and 0.2 M respectively. Table 1 shows the results of incubation of the above viruses in 10X MEM adjusted to pH 3.0 with Tris base, in viral diluent at pH 7.4, and in citrate-phosphate buffers at pH 3.0 and 8.0. The citrate-phosphate buffers and MEM were not viracidal for poliovirus, and poliovirus was not inactivated at pH 3.0; at pH 8.0, the citrate-phosphate buffer did not affect infectivity of rhinovirus type 14, but at pH 3.0 the rhinovirus was inactivated.

Effect of CsCl on Two Human Rhinoviruses

For a number of years, a standard procedure for the final step in the purification of picornaviruses and other non-enveloped viruses has been by isopycnic centrifugation
## TABLE 1

**EFFECT OF BUFFERS ON TWO HUMAN PICORNAVIRUSES**

<table>
<thead>
<tr>
<th></th>
<th>Time (hours)</th>
<th>Poliovirus Type 2</th>
<th>Rhinovirus Type 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.4(^a)</td>
<td>0</td>
<td>8.5(^d)</td>
<td>5.5(^d)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8.5</td>
<td>5.5</td>
</tr>
<tr>
<td>3.0(^b)</td>
<td>0</td>
<td>8.5</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8.0</td>
<td>&lt;1.5</td>
</tr>
<tr>
<td>7.4(^a)</td>
<td>0</td>
<td>2.4 x 10^6</td>
<td>2.9 x 10^6</td>
</tr>
<tr>
<td>3.0(^c)</td>
<td>3</td>
<td>2.2 x 10^6</td>
<td>&lt;1.0 x 10^4</td>
</tr>
<tr>
<td>8.0(^c)</td>
<td>0</td>
<td>2.8 x 10^6</td>
<td>2.1 x 10^6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.0 x 10^6</td>
<td>1.8 x 10^6</td>
</tr>
</tbody>
</table>

- **a** - Virus diluent.
- **b** - Eagle's minimal essential medium, adjusted to pH 3.0 with 1 M Tris base.
- **c** - Citrate-phosphate buffer.
- **d** - Titers expressed as TCID\(_{50}\)/ml.
- **e** - Titers expressed as PFU/ml.
at low temperatures (0° to 5° C) in cesium-chloride gradients. Centrifugation time intervals have varied from as short as 20 to as long as 100 hours. During the course of this investigation, two reports appeared in the literature (59, 94) suggesting that rhinoviruses are inactivated in CsCl solutions. Half of the infectivity was lost during isopycnic centrifugation of human rhinovirus types 2, 14, 20, and an equine rhinovirus (59). In light of this information, it was of interest to know if the rhinovirus used in these investigations was sensitive to CsCl. To this end it was decided to determine the infectivity of HRV-14 that had been stored in CsCl at -22° C for several years, and to determine the loss of infectivity of two rhinoviruses, types 14 and 20, after either storage or centrifugation for 48 hours in CsCl solutions at 0° C. Virus stored at 0° C in gradient diluent which was 0.01 M Tris buffer, pH 8.1 was used as a control.

Table 2 presents the results of the infectivity of HRV-14 after storage at -22° C in CsCl for as long as two years. The viral preparations were collected from CsCl gradients and stored at the density at which they were collected. The loss of infectivity with storage ranged from as low as approximately 20 per cent to as high as 80 per cent. The average loss, exclusive of the third sample listed in Table 2, was approximately 30 per cent. If the third sample is included, the average loss of infectivity
**TABLE 2**

**EFFECT OF CsCl ON INFECTIVITY OF HUMAN RHINOVIRUS TYPE 14 AFTER STORAGE AT -22° C**

<table>
<thead>
<tr>
<th>Density (g/cc)</th>
<th>PFU/ml after collection from gradient</th>
<th>Time</th>
<th>PFU/ml after storage</th>
<th>Per cent reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.41</td>
<td>$1.2 \times 10^8$</td>
<td>16</td>
<td>$8.6 \times 10^7$</td>
<td>28.3</td>
</tr>
<tr>
<td>1.44</td>
<td>$1.5 \times 10^7$</td>
<td>24</td>
<td>$9.5 \times 10^6$</td>
<td>36.6</td>
</tr>
<tr>
<td>1.39</td>
<td>$3.8 \times 10^6$</td>
<td>24</td>
<td>$8.5 \times 10^5$</td>
<td>77.6</td>
</tr>
<tr>
<td>1.39</td>
<td>$4.2 \times 10^6$</td>
<td>24</td>
<td>$3.3 \times 10^6$</td>
<td>21.4</td>
</tr>
</tbody>
</table>

a - Plaque forming units (PFU)/ml.

b - Represents only one determination of PFU/ml.

c - Storage time in months.

d - Two determinations of PFU/ml for all samples except the first one.
was approximately 40 per cent. The loss of infectivity observed for three out of the four preparations of HRV-14 could well be within the limits expected for viral decay under these conditions. Thus, human rhinovirus type 14 was found to be relatively stable after storage at -22°C for two years in CsCl solutions with densities between 1.39 g/cc and 1.45 g/cc.

The effects of isopycnic centrifugation and storage in cesium chloride on human rhinovirus types 14 and 20 are listed in Table 3. The mean density of the cesium chloride for viral preparations was 1.45 g/cc. Human rhinovirus type 20 (HRV-20) lost approximately 80 to 90 per cent infectivity after being stored or centrifuged in cesium chloride for 48 hours. Control preparations for this virus lost a similar amount of infectivity. This data suggests that inactivation of HRV-20 was temperature dependent and not necessarily cesium chloride dependent. Human rhinovirus type 14 lost approximately 67 per cent and 75 per cent of its infectivity following storage and centrifugation respectively in cesium chloride. The infectivity loss for control preparations of HRV-14 was approximately 20 per cent. It appears from this data that HRV-14 is inactivated to some extent in the presence of cesium chloride. Inactivation of HRV-14 was not dependent upon centrifugation; since in cesium-chloride solutions, inactivation also occurred in the absence of centrifugation.
TABLE 3

EFFECT OF CsCl ON INFECTIVITY OF TWO RHINOVIRUSES AFTER EITHER ISOPYCNIC CENTRIFUGATION OR STORAGE FOR 4.8 HOURS AT 0° C

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>PFU/ml of HRV-14</th>
<th>Condition</th>
<th>Per cent Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.8 x 10^6</td>
<td>NC^a-Tris^b</td>
<td>0.0</td>
</tr>
<tr>
<td>48</td>
<td>1.4 x 10^7</td>
<td>NC -Tris</td>
<td>22.2</td>
</tr>
<tr>
<td>48</td>
<td>6.1 x 10^5</td>
<td>NC -CsCl^c</td>
<td>66.1</td>
</tr>
<tr>
<td>48</td>
<td>4.5 x 10^5</td>
<td>Cd -CsCl</td>
<td>75.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PFU/ml of HRV-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>48</td>
</tr>
<tr>
<td>48</td>
</tr>
<tr>
<td>48</td>
</tr>
</tbody>
</table>

^a - Samples were not centrifuged.
^b - Tris base, 0.01 M and pH 8.1, was used as diluent.
^c - The density of cesium chloride (CsCl) was 1.45 g/cc and made with 0.01 M Tris base pH 8.1 as diluent.
^d - Samples were centrifuged for 4.8 hours at 175,296 g.
^e - Per cent reduction equals PFU/ml at time zero minus PFU/ml at 4.8 hours, divided by PFU/ml at time zero, times 100.
Effect of pH on the Inactivation of Human Rhinovirus Type 14

Cesium chloride purified HRV-14 and poliovirus type 2 were diluted 1:10 in 0.2 M citrate-phosphate buffers with pH values of 3.0, 3.4, 4.0, 4.4, 5.0, 5.4, 6.0, 7.0, and 8.0. Human rhinovirus type 14 cell lysates clarified by centrifugation were treated similarly, except at pH 3.0 and 3.4 the samples were diluted 1:2 instead of 1:10. In all experiments, 0.2 ml of virus at an approximate concentration of $2 \times 10^7$ to $2 \times 10^8$ PFU/ml was mixed with the appropriate volume of buffer.

Figure 1 shows the inactivation of purified rhinovirus as a function of pH during incubation at room temperature for two hours in the above buffers. From pH 6.0 to 8.0 no loss of infectivity was observed. Treatment at pH 5.4 resulted in a loss of more than 99 per cent of the infectivity. Maximum inactivation occurred at pH 3.4 and 3.0. Infectious virus was found to be present after two hours at all pH values above 3.4. No virus was detected at pH values lower than 3.4. There was no significant loss of poliovirus infectivity at any pH. When non-purified (clarified) rhinovirus was used in inactivation studies, the extent of inactivation was similar to that of purified virus. However, some infectious virus was detected at pH 3.4 and 3.0 (Figure 2).
FIGURE 1

INACTIVATION OF PURIFIED HUMAN RHINOVIRUS TYPE 14 AS A FUNCTION OF pH

Virus samples were diluted 1:10 in citrate-phosphate buffers ranging in pH from 3.0 to 8.0. After incubation for two hours at room temperature, the amount of residual virus was determined by plaque assay. Poliovirus was included as an example of an acid stable picornavirus and was treated like the rhinovirus. The virus concentrations (PFU/ml) at time zero and after two hours for poliovirus were $2.5 \times 10^6$ and $2.0 \times 10^6$. The corresponding rhinovirus concentrations were $2.2 \times 10^6$ and $1.8 \times 10^6$. 
FIGURE 1

POLOVIRUS

RHINOVIRUS

PFU/ml

<10^0

<10^1

<10^2

<10^3

<10^4

<10^5

<10^6

pH

3.0

4.0

5.0

6.0

7.0

8.0
Infected cell lysates were clarified by centrifugation at 900 g for ten minutes. Following clarification, virus samples were incubated for two hours in citrate-phosphate buffers ranging in pH from 3.0 to 8.0. Following incubation, the amount of infectious virus was determined by tube assay using ten tubes per dilution. The initial and final virus concentrations (TCID_{50}/ml) for controls were similar, 7.1 and 6.9 respectively.
Effect of Time on the Inactivation of Human Rhinovirus Type 14

Usually, the standard procedure for testing picornaviruses for acid lability involves incubating the candidate virus as well as a known positive and negative control virus at pH 3.0 or 5.0 for two to three hours at room temperature. Following incubation, the amount of infectious virus is assayed. To determine the effect of time on inactivation of human rhinovirus type 14, kinetic experiments were done at pH 3.0 and 5.0. Inactivation was initiated by making a 1:2 dilution of HRV-14 in 10X MEM (pH 3.0) or citrate-phosphate buffer (pH 5.0). To stop inactivation, the pH of the reaction mixture was raised above 6.0 by diluting 1:5 with pH 8.0 citrate-phosphate buffer. Figure 3 shows the inactivation of three different preparations of HRV-14 as a function of time. No loss of infectivity was seen at pH 7.0, while at pH 5.0 inactivation reached a maximum by 20 minutes with losses greater than 99 per cent of the infectivity. After 20 minutes, the amount of infectious virus present at pH 5.0 remained relatively constant. The slopes of the inactivation curves appear to be nearly linear during the early minutes of inactivation. With purified virus, the slope of the inactivation curve appears to be steeper. Similar kinetic experiments done at pH 3.0 revealed that inactivation was very rapid and infectious virus could not be detected.
Figure 3

Inactivation of Human Rhinovirus Type 14 at pH 5.0 as a Function of Time

Rhinovirus type 14 consisting of cell lysate samples (clarified by centrifugation), fluorocarbon-treated samples, or samples purified in cesium chloride gradients was inactivated by diluting the virus 1:2 in pH 5.0 citrate-phosphate buffer. At specified time intervals, the reaction was stopped by diluting aliquots 1:5 with pH 8.0 citrate-phosphate buffer. The amount of infectious virus present was determined by plaque assay. The initial virus concentration was between $5 \times 10^6$ and $6 \times 10^7$ PFU/ml.
FIGURE 3

Graph showing the decline of PFU/ml over time with different treatments:

- CONTROL
- CLARIFIED
- FLUOROCARBONED
- PURIFIED

The graph plots minutes on the x-axis and PFU/ml on the y-axis, with various points indicating the decline at different time intervals.
after one minute (Table 4). Viral samples treated at pH 5.0 by dialysis for one hour lose infectivity when the pH is lowered to 3.0 (Table 5).

Effect of Temperature on the Inactivation of Human Rhinovirus Type 14

Experiments involving the effect of temperature on the inactivation of HRV-14 at pH 5.0 revealed that inactivation was temperature dependent (Table 6). Virus incubated at 0°C for five minutes in pH 5.0 citrate-phosphate buffer showed no loss of infectivity. In contrast, virus incubated for five minutes at 24°C in the same buffer lost nearly 99 percent of its infectivity.

Effect of Acid Treatment on Recovery of Human Rhinovirus Type 14 Protein and Nucleic Acid

Purified HRV-14, labeled either with ³H-uridine or ³H-protein hydrolysate, was inactivated at pH 3.0 at room temperature. Inactivation was accomplished by adding N HCl. After two hours, samples were adjusted to neutral pH with N NaOH and then incubated with 10 µg/ml of RNase for 30 minutes at 37°C. Controls were treated similarly, but were not subjected to pH 3.0. After RNase treatment, samples were precipitated with TCA. Treatment at pH 3.0 had little effect on the recovery of viral proteins (Table 7). Over 90 percent of the viral proteins remained TCA precipitable following pH 3.0 treatment. RNase treatment
**TABLE 4**

LOSS OF INFECTIVITY OF HUMAN RHINOVIRUS TYPE 14 AT pH 3.0

Surviving Virus (PFU/ml)

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Control (pH 8.0)(^a)</th>
<th>Treatment (pH 3.0)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.8 x 10^7</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>&lt;1.0 x 10^1</td>
</tr>
<tr>
<td>5</td>
<td>2.5 x 10^7</td>
<td>&lt;1.0 x 10^1</td>
</tr>
</tbody>
</table>

\(a\) - Citrate-phosphate buffer.

\(b\) - Eagle's minimal essential medium, adjusted to pH 3.0 with 1.0 M Tris base.
TABLE 5

LOSS OF INFECTIVITY OF HUMAN RHINOVIRUS TYPE 14
AFTER LOWERING THE pH FROM 5.0 TO 3.0

Surviving Virus (PFU/ml)

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Control&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Treatment&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 8.0</td>
<td>pH 3.0</td>
</tr>
<tr>
<td>0</td>
<td>$1.3 \times 10^7$</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>$9.6 \times 10^6$</td>
<td>$&lt;1.0 \times 10^1$</td>
</tr>
</tbody>
</table>

<sup>a</sup> - Virus was plaque assayed at time zero and then again after dialysis for two hours at room temperature against pH 8.0 citrate-phosphate buffer.

<sup>b</sup> - A viral sample was divided into two aliquots; one aliquot was plaque assayed after dialysis for one hour at room temperature against pH 5.0 citrate-phosphate buffer. The second aliquot was dialyzed for one hour at room temperature against pH 5.0 citrate-phosphate buffer, followed by dialysis for one hour at pH 3.0.
TABLE 6

EFFECT OF TEMPERATURE ON INFECTIVITY OF
HUMAN RHINOVIRUS TYPE 14 AT pH 5.0

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Control(a)</th>
<th>Treated(b)</th>
<th>Per cent Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0° C</td>
<td>3.8 x 10^7</td>
<td>3.9 x 10^7</td>
<td>0.0</td>
</tr>
<tr>
<td>24° C</td>
<td>3.2 x 10^7</td>
<td>3.8 x 10^5</td>
<td>98.8</td>
</tr>
</tbody>
</table>

\(a\) - Initial viral concentration in pH 7.0 citrate-phosphate buffer at 0° C and 24° C at time zero.

\(b\) - Virus was incubated in pH 5.0 citrate-phosphate buffer for five minutes at 0° C and 24° C.
### TABLE 7
EFFECT OF ACID TREATMENT ON HUMAN RHINOVIRUS TYPE 14 PROTEIN

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C.P.M.</th>
<th>Per cent Recovery of Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>320</td>
<td>100</td>
</tr>
<tr>
<td>pH 3.0</td>
<td>300</td>
<td>93.8</td>
</tr>
<tr>
<td>pH 3.0 + RNase</td>
<td>285</td>
<td>89.1</td>
</tr>
<tr>
<td>RNase only</td>
<td>294</td>
<td>91.8</td>
</tr>
</tbody>
</table>

\(a\) - Counts per minute (C.P.M.) corrected for background.
alone or pH 3.0 and RNase treatment resulted in 91 and 89 per cent recovery respectively of viral proteins. Only 66 per cent of the viral RNA could be recovered by TCA precipitation following pH 3.0 treatment of 3H-uridine-labeled HRV-14 (Table 8). Ribonuclease converted greater than 98 per cent of the RNA from acid-treated viruses to an acid soluble form. RNase treatment alone did not significantly affect the recovery of RNA from virions not exposed to pH 3.0.

**Cesium Chloride and Sucrose Gradient Analysis of Acid-Treated and Non-treated Radioactive Rhinovirus Type 14**

Figure 4 shows infectivity and radioactivity profiles in a sucrose gradient of non-treated 3H-valine-labeled HRV-14. Poliovirus type 2 yielded similar profiles in sucrose gradients (Figure 6). These results demonstrate that the virions isolated from sucrose gradients after purification in a CsCl gradient are intact and infectious. No material was observed in gradients where empty capsids (75 to 80S material) would be expected to sediment, indicating that a relatively pure and homogeneous preparation of HRV-14 was obtained by isopycnic centrifugation in CsCl gradients.

Rhinovirus type 14 with either tritium-labeled-protein or nucleic acid was acid treated at pH 3.0. Following acid treatment, samples were subjected to CsCl and/
TABLE 8

RELEASE OF RNA FROM HUMAN RHINOVIRUS TYPE 14
VIRIONS AFTER ACID TREATMENT

<table>
<thead>
<tr>
<th></th>
<th>C.P.M. $^a$</th>
<th>Per cent Recovery of Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>641</td>
<td>100</td>
</tr>
<tr>
<td>pH 3.0</td>
<td>425</td>
<td>66.3</td>
</tr>
<tr>
<td>pH 3.0 + RNase</td>
<td>14</td>
<td>2.2</td>
</tr>
<tr>
<td>RNase only</td>
<td>634</td>
<td>98.9</td>
</tr>
</tbody>
</table>

$a$ - Counts per minute (C.P.M.) corrected for background.
FIGURE 4

RADIOACTIVITY AND INFECTIVITY PROFILES OF HUMAN RHINOVIRUS TYPE 14 IN A SUCROSE GRADIENT

$^3$H-valine-labeled HRV-14 was purified in cesium chloride-isopycnic gradients. After removal of cesium by dialysis, 100 lambda samples were layered on 15 to 30 per cent linear sucrose gradients. Following sedimentation velocity centrifugation, 0.5 ml fractions were collected and analyzed for radioactivity. Infectivity was determined by plaque assay.
FIGURE 4

![Graph showing fraction of 3H CPM against fraction and percentage infectivity.](image-url)
or sucrose gradient analysis. Figure 5 shows a radioactive profile in a sucrose gradient of \(^3\text{H}\)-protein hydrolysate labeled rhinovirus type 14 that was treated at pH 3.0 for 15 minutes at room temperature in 10X MEM made 1.0 per cent with 2-ME. After treatment, samples were adjusted to 7.0 with \(\text{N} \ \text{NaOH}\). \(^{14}\text{C}\)-leucine-labeled HRV-14 was added that contained CsCl gradient purified empty capsids and infectious virus as markers. Centrifugation was for two hours at 41,000 RPM (286,434 g) in an SW 41 rotor at 5° C. It is apparent from these results that infectious virus was destroyed following acid treatment at pH 3.0. In addition, no material was evident after acid treatment which had an S value similar to empty capsids.

When \(^3\text{H}\)-protein hydrolysate labeled rhinovirus type 14 was exposed to citrate-phosphate buffer (pH 3.0) in the absence of 2-ME and not adjusted to neutral pH, sedimentation profiles as presented in Figure 6 were observed. Twelve ml, 15 to 30 per cent sucrose gradients were used, and centrifugation was at 41,000 RPM (286,434 g) for 110 minutes in an SW 41 rotor at 5° C. Poliovirus labeled with \(^{14}\text{C}\)-leucine was added as a marker and allowed to co-sediment with the treated sample. Under these conditions, no viral material was seen in the gradients where infectious (150-160S) and non-infectious (75-80S) particles would be expected to be found. Nearly 50 per cent of the radioactive material pelleted following this type of acid treatment.
Purified HRV-14 samples labeled with $^3$H-protein hydrolysate were inactivated at pH 3.0 in 10X MEM containing 1.0 percent 2-ME. After inactivation, samples were adjusted to pH 7.0 and then centrifuged in 15 to 30 percent linear sucrose gradients for 110 minutes at 286,434 g in an SW 41 rotor at 5$^\circ$C. $^{14}$C-leucine-labeled HRV-14 was added to the gradient as a marker. Following centrifugation, approximately 0.5 ml fractions were collected, precipitated with trichloroacetic acid onto filters, and then assayed for radioactivity.
Purified HRV-14, labeled with $^3$H-protein hydrolysate was inactivated at pH 3.0 in citrate-phosphate buffer and then allowed to co-sediment in 15 to 30 per cent linear sucrose gradients with $^{14}$C-leucine-labeled poliovirus. Centrifugation was for two hours at 26,434 g in an SW 41 rotor at 5° C. Following centrifugation, approximately 0.5 ml fractions were collected, precipitated with trichloroacetic acid onto filters, and then assayed for radioactivity.
FIGURE 6.

- **14C-POLIOVIRUS**
- **TREATED \(^3\)H-RV-14**

- PELLET
Pellets were resuspended by vigorously washing the bottom of the centrifuge tubes with approximately 0.3 ml of water.

$^3$H-protein hydrolysate labeled rhinovirus type 14, having a density of 1.30 g/cc, was acid treated in citrate-phosphate buffer (pH 3.0) in the absence of 2-ME and then centrifuged in 15 to 30 per cent linear sucrose gradients. The results of a typical experiment are given in Figure 7. No radioactive peak corresponding to empty capsids (75 to 80S material) was obtained. A large portion of the radioactivity pelleted at the bottom of the gradient.

Figure 8 shows the CsCl density gradient centrifugation of $^3$H-protein hydrolysate labeled rhinovirus type 14 that was inactivated at pH 3.0 for two hours at room temperature. An acid environment was achieved by adding N HCl. After acid treatment, samples were adjusted to pH 7.0 with N NaOH. Non-treated virus banded at a density of 1.40 g/cc. Nearly all of the radioactivity was located in this peak. Radioactivity from acid-treated virus was found at the top of the CsCl gradients at a density of approximately 1.33 g/cc. This data suggests that not only is there a change in density following acid treatment, but also a possible change from an infectious to a non-infectious state. Figure 9 shows the results of CsCl density centrifugation of $^3$H-uridine-labeled rhinovirus that was acid treated at pH 3.0 as mentioned above. Following acid treatment, the viral samples were adjusted to
FIGURE 7

SUCROSE-SEDIMENTATION PROFILE OF ACID-TREATED TRITIATED HUMAN RHINOVIRUS TYPE 14 EMPTY CAPSIDS

Empty capsids of HRV-14 labeled with $^3$H-protein hydrolysate were acid treated at pH 3.0 in citrate-phosphate buffer and then centrifuged for 16 hours at 133,492 g in an SW 41 rotor at 5°C. Non-treated samples were centrifuged for two hours at 286,434 g in an SW 41 rotor at 5°C.
FIGURE 8

DENSITY GRADIENT CENTRIFUGATION OF ACID-TREATED HUMAN RHINOVIRUS TYPE 14 LABELED WITH \(^{3}H\)-PROTEIN HYDROLYSATE

Purified HRV-14 samples labeled with \(^{3}H\)-protein hydrolysate were treated at pH 3.0, adjusted to pH 7.0, and then subjected to isopycnic centrifugation for 60 hours at 175,296 g in an SW 39 rotor at 5° C. Following centrifugation, approximately 0.4 ml fractions were collected and assayed for radioactivity.
FIGURE 9

DENSITY GRADIENT CENTRIFUGATION OF ACID-TREATED HUMAN RHINOVIRUS TYPE 14 LABELED WITH $^3$H-URIDINE

Purified HRV-14 samples labeled with $^3$H-uridine were treated at pH 3.0, adjusted to pH 7.0, and then subjected to isopycnic centrifugation for 60 hours at 175,296 g in an SW 39 rotor at 5° C. Following centrifugation, approximately 0.4 ml fractions were collected and assayed for radioactivity.
% RECOVERED RADIOACTIVITY

UNTREATED

TREATED

DENSITY

1.55 1.50 1.45 1.40 1.35 1.30
pH 7.0 by adding NaOH. With non-treated virus, radioactivity was again located in a peak having a density of 1.40 g/cc. Acid treatment resulted in radioactivity being scattered throughout the gradient with a peak at a density of 1.42 g/cc.

**Polyacrylamide Gel Electrophoresis (PAGE) of Acid-Treated and Non-treated Human Rhinovirus Type 14**

To insure the reproducibility of the SDS-PAGE system used in these studies, poliovirus, rhinovirus, and rhinovirus material having the density of empty capsids were subjected to SDS-PAGE in 10 per cent gels. The results were compared with those previously reported in the literature. Figure 10 represents a typical electrophoretic profile of $^{14}C$-leucine-labeled poliovirus. All four viral structural polypeptides (VP 1, 2, 3, and 4) are present. The viral structural polypeptides (VP's) are identified numerically by assigning the slowest component as VP 1, et cetera. For comparative purposes, Table 9 includes molecular weights of viral polypeptides observed during this study, and as reported in the literature (46). When purified-infectious rhinovirus type 14 was treated similarly, four structural components were observed (Table 9 and Figure 13). Figure 11 gives a plot of the relative migrations of rhinovirus structural proteins and known molecular weight protein standards of bovine serum albumin.
Purified $^{14}\text{C}$-leucine-labeled poliovirus was degraded in the presence of 1.0 per cent SDS, 1.0 per cent 2-ME, 6 M urea, and heat. Following degradation, 200 lambda samples were electrophoresed on 10 per cent polyacrylamide gels, 80 mm in length, for seven hours at room temperature. Fractions 1.0 mm in length were collected, following electrophoresis, and assayed for radioactivity. The origin (cathode) was always at the left, and polypeptide migration was always to the right towards the anode.
### TABLE 9

**MOLECULAR WEIGHTS OF POLIOVIRUS TYPE 2 AND RHINOVIRUS TYPE 14 POLYPEPTIDES**

<table>
<thead>
<tr>
<th>Poliovirus Type 2</th>
<th>Molecular Weight</th>
<th>Apparent Molecular Weight</th>
<th>Per cent Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP 0(^a)</td>
<td>-</td>
<td>444,000</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>32,000(^b)</td>
<td>36,000</td>
<td>12.5</td>
</tr>
<tr>
<td>2</td>
<td>25,000</td>
<td>29,500</td>
<td>18.0</td>
</tr>
<tr>
<td>3</td>
<td>24,000</td>
<td>26,500</td>
<td>10.4</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>4,700</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rhinovirus Type 14</th>
<th>Molecular Weight</th>
<th>Apparent Molecular Weight</th>
<th>Per cent Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP 0(^a)</td>
<td>455,000(^c)</td>
<td>40,000</td>
<td>11.1</td>
</tr>
<tr>
<td>1</td>
<td>42,000</td>
<td>36,000</td>
<td>14.2</td>
</tr>
<tr>
<td>2</td>
<td>33,000</td>
<td>29,500</td>
<td>10.6</td>
</tr>
<tr>
<td>3</td>
<td>30,000</td>
<td>27,500</td>
<td>8.3</td>
</tr>
<tr>
<td>4</td>
<td>6,000</td>
<td>7,600</td>
<td>26.6</td>
</tr>
</tbody>
</table>

\(^a\) - Infectious virions contain viral polypeptides (VP) VP 1 to 4, while naturally occurring empty capsids have VP 0, 1, and 3.

\(^b\) - Molecular weights of poliovirus type 2 polypeptides as reported by Holland, J. J. and Kiohn, E. D. (46).

\(^c\) - Molecular weights of rhinovirus type 14 polypeptides as reported by Korant et al. (59).

\(^d\) - Per cent deviation determined by molecular weight minus apparent molecular weight, divided by molecular weight, times 100.
FIGURE 11

RELATIVE MIGRATION OF MOLECULAR WEIGHT PROTEIN STANDARDS AND TRITIATED HUMAN RHINOVIRUS TYPE 14 STRUCTURAL PROTEINS

Samples were treated with 1.0 per cent SDS, 1.0 per cent 2-ME, 6 M urea, and heat. After treatment, samples were electrophoresed on 10 per cent polyacrylamide gels, 220 mm in length, for 18.5 hours at room temperature. Protein standards were identified by staining with 0.25 per cent Coomassie blue, while viral polypeptides (VP's) were verified by slicing the gels into 1.0 mm sections and assaying for radioactivity. The protein standards and their molecular weights were: bovine serum albumin (BSA), 67,000 daltons; *B. subtilis* alpha amylase, 48,960 daltons; yeast alcohol dehydrogenase, 37,000 daltons; and bovine trypsin, 23,300 daltons.
(BSA), alpha amylase, alcohol dehydrogenase, and trypsin. From this plot of molecular weight versus relative migration, an estimate of molecular weights of the rhinovirus structural proteins was obtained. Furthermore, poliovirus type 2 labeled with $^{14}$C-leucine and $^{3}$H-protein hydrolysate labeled rhinovirus type 14 were co-electrophoresed at pH 6.8 (Figure 12). This was done to also get an estimation of the molecular weights of the rhinovirus polypeptides. In this experiment, VP 2 and 3 from the rhinovirus were not resolved, however, VP 1 was always found to be smaller in molecular weight than the VP 1 of poliovirus.

Rhinovirus empty capsid-like material was found to have a density of 1.30 g/cc (Table 10) and contained VP 0, 1, and 3. The relative mobility of these polypeptides and their molecular weights are given in Figure 11 and Table 9 respectively.

Rhinovirus type 14 samples labeled with $^{3}$H-protein hydrolysate were treated at pH 3.0 in citrate-phosphate buffer. Following acid treatment, samples were degraded with SDS, 2-ME, and heat. Controls were handled similarly except for acid treatment. These experiments revealed no difference in electropherograms between acid-treated and non-treated samples (Figure 13). When acid-treated and non-treated samples were analyzed in non-detergent gels, a distinct difference between the two were noted (Figure 14). Non-treated samples did not penetrate 5.0 per cent
Purified virus samples were degraded in the presence of 1.0 per cent SDS, 1.0 per cent 2-ME, 6 M urea, and heat. Following treatment, 200 lambda samples were co-electrophoresed on 10 per cent polyacrylamide gels, 80 mm in length, for seven hours. Radioactivity was measured in fractions sliced 1.0 mm in length.
FIGURE 12

- $^{14}$C POLIOVIRUS TYPE 2
- $^{3}$H RHINOVIRUS TYPE 14
TABLE 10

DENSITY DETERMINATION OF HUMAN RHINOVIRUS
TYPE 14 EMPTY CAPSIDSa

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>CPM/100 lambdab</th>
<th>Density (g/cc)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>4,240</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>5,600</td>
<td>1,311</td>
</tr>
<tr>
<td>9</td>
<td>8,040</td>
<td>1,307</td>
</tr>
<tr>
<td>10</td>
<td>11,740</td>
<td>1,304</td>
</tr>
<tr>
<td>11</td>
<td>10,360</td>
<td>1,301</td>
</tr>
<tr>
<td>12</td>
<td>8,520</td>
<td>1,294</td>
</tr>
<tr>
<td>13</td>
<td>7,480</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>6,840</td>
<td>-</td>
</tr>
</tbody>
</table>

a - Fractions from three cesium-chloride gradients, ranging in density from 1.27 g/cc to 1.34 g/cc, were pooled and recentrifuged at 39,000 RPM (175,296 g) for 24 hours in an SW 39 rotor at 5°C. After centrifugation, approximately 0.2 ml fractions were collected and assayed for radioactivity.

b - Counts per minute (CPM) were determined by assaying the radioactivity obtained after trichloroacetic acid precipitation of five lambda samples.

c - Densities were determined gravimetrically at room temperature.
FIGURE 13

POLYPEPTIDE COMPOSITION OF ACID-TREATED AND NON-TREATED HUMAN RHINOVIRUS TYPE 14

Purified HRV-14 labeled with $^3$H-protein hydrolysate was either acid treated or non-treated; and then degraded with 1.0 per cent SDS, 1.0 per cent 2-ME, and heat. Samples were run on 10 per cent polyacrylamide gels, 220 mm in length, for 18 1/2 hours at room temperature. Following electrophoresis, fractions 1.0 mm in length were collected and assayed for radioactivity.
FIGURE 14

POLYACRYLAMIDE GEL ELECTROPHORESIS OF ACID-TREATED AND NON-TREATED TRITIATED HUMAN RHINOVIRUS TYPE 14

Purified HRV-14 labeled with $^3$H-valine was treated at pH 3.0 or not treated; and then subjected to electrophoresis on 5.0 per cent non-detergent gels. Following electrophoresis, gels were sliced into 1.0 mm fractions and assayed for radioactivity.
**Figure 14**

- **NON TREATED**
- **pH 3.0 TREATED**

Graph showing CPM (Counts Per Minute) against Fraction.
gels and remained at the origin. In contrast, acid-treated samples penetrated the gels and formed one major rapidly migrating component and another component that remained near the origin. Attempts to further characterize the rapidly migrating component released by acid treatment using gels of at least three different concentrations have failed.
DISCUSSION

Picornaviruses are generally stable in solutions of high molarity if the pH is appropriate. For example, certain picornaviruses are stable in 1.0 M MgCl$_2$ and 2.0 M NaCl (65, 89). In 3.2 M solutions of CsCl (density 1.40 g/cc) at 0°C, rhinovirus type 14 was observed to lose 65 and 75 per cent of its infectivity after 48 hours incubation or centrifugation (Table 3). Storage of rhinovirus type 14 at -22°C in cesium-chloride solutions, with densities of 1.39 g/cc to 1.44 g/cc, had no appreciable affect on infectivity (Table 2). During purification procedures, prolonged contact of rhinovirus type 20 with cesium chloride resulted in 50 to 90 per cent loss of infectivity (94). Electron microscopic examination of these cesium-chloride purified type 20 particles revealed that phosphotungstic acid (PTA), infiltrated 30 to 50 per cent of the intact virions. It has been assumed that cesium-chloride purified virions are apparently homogeneous components. The 30 to 50 per cent infiltration of intact type 20 rhinovirus virions with PTA suggests either a lack of homogeneity, or some differential inactivation of the virus. Poliovirus prepared under similar conditions as 84.
rhinovirus type 20 showed no significant loss in infectivity or increase in PTA filled particles (94).

In this laboratory, rhinovirus type 20 after storage at 0°C for 48 hours was found to lose similar amounts of infectivity (80 to 90 per cent) whether the virus was in cesium chloride or not. Korant et al. (59) found that about half of rhinovirus infectivity was lost during isopycnic banding in cesium chloride. Thomas and co-workers (105) were able to recover between 50 and 80 per cent of rhinovirus infectivity during the course of concentrating and purifying rhinovirus type 14. No loss of infectivity was observed when several rhinovirus strains were diluted in cesium chloride, potassium tartrate, or potassium citrate and then held at 4°C overnight (72). After isopycnic centrifugation for 70 hours at 4°C, 18 per cent of the input infectivity was recovered for rhinovirus type 1A (22). Rhinovirus type 2, strain HGP, has been reported to be unstable in cesium-chloride solutions (13).

It appears that rhinoviruses lose some infectivity upon centrifugation in cesium-chloride gradients. The loss of infectivity of rhinoviruses in cesium chloride might be correlated with the accessibility and binding of cesium ions by these viruses. However, since this procedure yields relatively pure and highly infectious preparations, the loss of infectivity which occurs does not justify discontinuing the use of cesium-chloride gradients for purifica-
In this study it has been shown that rhinoviruses do not lose infectivity at pH 6.0, but at pH 5.4 greater than 99 per cent of the initial infectivity is lost. As the pH decreased from 5.4 to 3.0 so did infectivity. Dimmock and Tyrrell (24) found that seven different rhinovirus strains lost from 1.3 to 3.0 log_{10} of infectivity after incubation in pH 5.0 to 5.4 buffer for 60 minutes at 37° C. Under the same conditions, these same investigators found at pH 4.3 to 4.5 greater than 2.1, 2.6, 3.8, and 4.5 log_{10} reduction in infectivity with four different rhinoviruses. During the present study, greater than a 4.0 log_{10} reduction in infectivity was consistently observed after treatment of human rhinovirus type 14 at pH 4.0.

Kinetic experiments demonstrated for the first time, that incubation of rhinovirus type 14 at pH 3.0 for one minute destroyed all evidence of infectivity. At pH 5.0, inactivation reached a maximum after 20 minutes of incubation. The amount of infectious virus present after two hours incubation at pH 5.0 was similar to that at 20 minutes. The reason for the existence of a surviving virus population after acid treatment at pH 5.0 is not known. Such a surviving population of virus was observed at pH 5.0 with both purified and non-purified virus preparations.

Experiments showing the inactivation of purified rhinoviruses as a function of pH suggest that residual virus
may be present after all treatments above approximately pH 3.4. It appears that the surviving fraction after acid treatment decreases as pH decreases. The surviving fraction might be the result of viral aggregation. Viral aggregates may be formed by precipitation of virus at low pH. Viral particles close to the center of aggregates may be protected from the acid environment. When viral samples treated for one hour at pH 5.0 were lowered to pH 3.0, no infectious virus could be detected. Aggregate formation may be reversed by the latter conditions with the end result being a loss in infectivity.

At 0°C, no loss of infectivity of rhinoviruses was observed after treatment at pH 5.0 for five minutes. A similar dependence of inactivation on temperature has been reported for the cardioviruses (65, 88). ME-virus did not lose any infectivity after acid treatment for ten minutes at 0°C, while at 37°C under the same conditions greater than 90 per cent of the infectivity was lost (89). At 7°C Mengo virus lost less than 10 per cent infectivity after 90 minutes of acid treatment, while at 37°C greater than 99.9 per cent infectivity was lost over the same time interval (65). Although rhinovirus and cardiovirus inactivation is temperature dependent, the latter is also dependent upon specific halide concentrations. Since the inactivation rate of rhinoviruses also appears to be temperature dependent, the degree of inactivation should
be controllable by adjusting time, temperature, or pH.

In poliovirus-infected cells, two kinds of particles are produced, infectious virions with a sedimentation value of 150 to 160S and empty capsids with a sedimentation value of 73 to 75S (69, 87, 92). Acid treatment has no effect on infectious poliovirus, but when infectious poliovirus is treated at a pH of 10.5, empty capsids are formed. These empty capsids lack nucleic acid and VP I (62). Korant et al. (59) found that when rhinoviruses were treated at pH 5.0, followed by adjustment to pH 7.0, two components were produced and each lacked VP 4. The sedimentation values of these components in sucrose gradients were estimated to be 135S and 75S respectively. One of these components, the "A" particle, was associated with RNA.

In the present study, acid inactivation of rhinovirus type 14 at pH 3.0 resulted in the complete destruction of the integrity of the virion. After acid inactivation, material was observed near the top and at the bottom of sucrose gradients. The material at the bottom of sucrose gradients had an S value greater than infectious virus. No empty capsid-like material was formed following treatment at pH 3.0. In fact, the same acid treatment of empty capsid-like material resulted in its destruction. In addition, PAGE of pH 3.0 treated and non-treated rhinoviruses revealed no differences in their polypeptide makeup. Before and after pH 3.0 treatment, VP 1 to 4 were
present in the same proportions.

It appears that the integrity of the FMDV virion also is destroyed after acid inactivation at pH 6.0. Treatment of FMDV at pH 6.0 to 6.5 leads to the production of 12 to 14S protein subunits and pelleted material (10, 21). Naturally occurring empty capsids of FMDV are stable at pH 6.0 (35). When two cardioviruses, ME- and Mengo viruses, were inactivated under acid conditions, pelleted material and protein subunits having a sedimentation value of approximately 14S were produced (43, 65, 66, 90). These 14S subunits were found to lack VP 4 and had a molecular weight of 400,000 to 420,000 daltons (79, 90). For ME-virus the 14S subunits existed as pentamers (29). Upon treatment with 1.0 to 2.0 M urea, these pentamers dissociated into five 5S subunits. Each of the 5S subunits consisted of one each of the non-identical viral polypeptides (VP 1, 2, and 3). Thus acid inactivation of rhinoviruses, FMDV, and cardioviruses leads to the formation of components in sucrose gradients that are light and pelleted.

After acid inactivation of Mengo virus variants, the major structural polypeptides were found in the pelleted material (80). An estimation of the S value or analysis of the structural polypeptides present in the pelleted material produced upon acid inactivation of HRV-14 reported here has not been determined. If rhinovirus inactivation behaves like FMDV and cardiovirus, one would expect similar
Following isopycnic centrifugation of pH 5.0 treated HRV-2, HRV-14, and an equine rhinovirus, Korant and co-workers (59) found an artificial dense component that was 0.04 to 0.05 g/cc greater in density than infectious virus. The yield of dense component for HRV-2 was approximately 40 per cent of the initial material, and was in a highly aggregated state. The yield of dense component for HRV-14 was poor and variable. In the present study, when $^3$H-uridine-labeled rhinovirus type 14 was inactivated at pH 3.0, adjusted to pH 7.0, and subjected to equilibrium isopycnic centrifugation, radioactivity was found throughout the gradient with a peak of radioactivity at a density of 1.42 g/cc. This dense component may be similar to the dense component produced after pH 5.0 treatment of HRV-2, HRV-14, and ERV as reported by Korant et al. (59).

The isopycnic centrifugation of radioactive amino acid labeled rhinovirus, previously inactivated at pH 3.0 and adjusted to pH 7.0, resulted in the localization of nearly all of the radioactivity at the top of the gradient at a density of 1.33 g/cc. This is in contrast to the results of Korant et al. (59) in which pH 5.0 treatment of rhinoviruses resulted in the production of two components with densities of 1.29 g/cc and 1.31 to 1.40 g/cc respectively (59). It is not known if these differences are the result of different pH values at which the two
studies were carried out or the buffers used.

In this study, polyacrylamide gel electrophoresis (PAGE) of SDS-treated poliovirus type 2 revealed the presence of four non-identical structural polypeptides (VP 1 to 4). The molecular weights in daltons for these structural components were 36,000, 29,500, 26,500, and 4,700. This is in agreement with that reported earlier by Holland and Kiehn (46). In the present study, purified preparations of poliovirus type 2 also were found to contain trace amounts of a non-capsid protein, NCVP6 (VP 6). Maizel et al. (62) suggested that the presence of NCVP6 in purified preparations was due to contamination with empty capsids.

When rhinovirus type 14 was degraded and analyzed on polyacrylamide gels, four non-identical structural polypeptides were observed (VP 1 to 4), Figure 13. The molecular weights in daltons for these structural components were found to be 36,000, 29,500, 27,500, and 7,600 respectively. These results are in agreement with those reported for type 14 (57, 59), and are similar to those reported for rhinovirus type 1A (74). When 14C poliovirus type 2 and 3H-IRV-14 were co-electrophoresed (Figure 12), rhinovirus VP 1 and 2 always migrated faster than poliovirus VP 1 and 2. This suggests that rhinovirus type 14 VP 1 and 2 may not be as large as previously reported (57, 59).

During this study, PAGE analysis of rhinovirus
protein having a density of empty capsids revealed the presence of VP 0, 1, and 3. Their respective molecular weights in daltons were 40,000, 36,000, and 27,500 and these are similar to those reported previously (59).

Current investigations of acid-treated and non-treated rhinovirus type 14 samples, electrophoresed on 10 per cent polyacrylamide gels containing SDS, revealed no differences. This suggests that inactivation at pH 3.0 does not result in any change or loss in the major structural polypeptides of the virus. By some mechanism, an acid environment destroys the infectivity, density, and structural integrity of the virus, but does not destroy the individual structural polypeptides. When samples were analyzed on non-detergent polyacrylamide gels, major differences were observed in the migration of viral components. Control samples remained essentially at the origin, while treated samples formed at least one major rapidly migrating component. This major component may represent the viral capsomers. Other investigations have involved electrophoretic studies using polyacrylamide gels containing detergent. These conditions do not allow for the determination of the basic component(s) formed after acid treatment. Using nondissociating PAGE conditions, Dunker and Rueckert (29) were able to provide strong evidence that the 5S component was the smallest single basic subunit of ME-virus.
SUMMARY

Infectivity of human rhinovirus type 14 was found to decrease below pH 6.0 and reached a maximum around pH 3.4. Maximum inactivation occurred within 20 minutes at pH 5.0 whereas at pH 3.0 inactivation was complete before one minute. A surviving viral population was observed as long as two hours following inactivation at pH 5.0. This surviving fraction was eliminated when the pH was lowered to 3.0.

Sucrose gradient analysis of pH 3.0 treated virus showed radioactive viral protein both at the top and bottom of gradients. Viral material at the bottom of sucrose gradients had a sedimentation value greater than infectious virus (150 to 160S). No 75 to 80S non-infectious viral particles could be detected. Following acid treatment, no viral material banding at the density of infectious virus could be found. Viral RNA was distributed throughout cesium-chloride gradients with a peak at a density of about 1.42 g/cc. Recovery of trichloroacetic acid precipitable viral proteins after acid treatment was around 90 per cent. This suggests little or no loss of viral proteins. After acid treatment, approximately 66 per
cent of viral RNA was recovered as trichloroacetic acid precipitable material. Ribonuclease treatment of acid-treated rhinovirus converted greater than 98 per cent of the RNA to acid soluble material, but did not appreciably affect the RNA of non-treated virions.

No differences could be detected after polyacrylamide gel electrophoresis of acid-treated and non-treated viral samples that were degraded in the presence of 1.0 per cent sodium dodecyl sulfate, 1.0 per cent 2-mercaptoethanol, 6 M urea, and heat. Polyacrylamide gel electrophoresis, in non-detergent gels, of virus treated at pH 3.0 with 2-mercaptoethanol revealed viral components that migrated into gels while untreated virus remained at the origin. The viral components entering the non-detergent gels are thought to be viral capsomers. These results imply that acid treatment of rhinovirions destroys infectivity, density, and structural integrity; but does not destroy the individual polypeptides of the virus.
APPENDIX A

Media

The preparation and use of growth and maintenance media for HeLa cells are essentially that described by Conant and Hamparian (17) and Thomas et al. (105).

A. Growth Medium. This medium was used for growing HeLa cells on glass in 32 ounce prescription bottles, three ounce prescription bottles, and tubes. This medium was prepared by adding the following ingredients to 810 ml of sterile distilled-demineralized water.

- Chlortetracycline•HCl: 50,000 µg
- Polymyxin B: 10,000 µg
- Streptomycin sulfate: 100,000 µg
- Potassium penicillin G: 100,000 units
- Earle's balanced salt solution, 10X with phenol red (Grand Island Biological Company, GIBCO, Grand Island, New York): 100 ml
- Amino acids, 50X in EMEM - minimum essential medium by Eagle (GIBCO): 20 ml
- Vitamins, 100X in EMEM (GIBCO): 10 ml
- 200 mM glutamine (GIBCO): 10 ml
- Fetal bovine serum (Flow Lab., Inc., Rockville, Maryland): 100 ml
- 7.5 per cent sodium bicarbonate: 20 ml

B. Spinner Medium. For growth of HeLa cells in suspension culture(s), spinner medium was prepared by adding the following ingredients to 810 ml of sterile distilled-
demineralized water.

Chlortetracycline·HCl 50,000 µg
MEM, Joklik-modified powdered preparation, 10X (GIBCO) 100 ml
Fetal bovine serum (Flow Lab., Inc.) 50 ml
Methocel, 4.0 per cent (Dow Chemical Company, Midland, Michigan) 20 ml

C. Maintenance Medium. This medium was used to maintain infected and uninfected HeLa cell monolayers. It was prepared the same way as growth medium except that it was buffered with 0.002 M Tris base, the fetal bovine serum concentration was decreased to 50 ml, and the sodium bicarbonate was increased to 25 ml.

D. Maintenance Media - For Incorporation of Radioactive Compounds into Viruses.

1. For reconstituted $^3$H- or $^{14}$C-protein hydrolysate (Appendix C) incorporation into viral protein, maintenance medium was prepared by reducing the amount of amino acids in a liquid EMEM preparation such that one-tenth the regular amount of amino acids were present. Also the sodium bicarbonate was increased to 4.0 ml.

2. For individual $^3$H- or $^{14}$C-amino acid incorporation (Appendix C), the medium was Eagle's minimum essential medium, Joklik-modified for suspension cultures (GIBCO), having the appropriate amino acid concentration reduced to one-tenth the normal amount. This medium contained 5.0 per cent fetal bovine serum (Flow Lab., Inc.) and 20 ml of 4.0 per cent methocel (Dow Chemical Company).
When used for monolayers this maintenance medium was buffered with 0.002 M Tris base, the methocel was omitted, the amount of sodium bicarbonate was increased to 2.2 g/liter, and 0.2 g/liter of calcium chloride was added. A liquid minimum essential medium (MEM) having Earle's balanced salt solution, glutamine, and sodium bicarbonate (at the same concentrations as maintenance media listed above), but completely deficient for L-leucine, was also used. Before use the medium was supplemented with 5.0 per cent fetal bovine serum (Flow Lab., Inc.), the antibiotics used for growth medium, Tris base to 0.002 M, and one-tenth the normal amount of L-leucine.

E. Overlay Media and Materials for Plaque Assay.

1. Double Strength Ionagar Number Two. Ionagar number two (Colab Laboratories, Chicago Heights, Illinois) was mixed 1:1 with double strength EMEM. Double strength EMEM was prepared the same was as growth medium EMEM (Appendix A), but with half as much water, 4.0 per cent fetal bovine serum, and 3.0 g/liter of sodium bicarbonate. The agar concentration for single strength ionagar number two was 0.6 and 1.0 per cent for the initial and final overlay media, respectively. Double strength ionagar number two was prepared by making a 1.2 and 2.0 per cent concentration for the initial and final overlay media, respectively. All agar preparations were made with distilled-demineralized water and then autoclaved for three minutes.
at 121° C. The agar solutions were cooled to 56° before diluting with 2X EMEM and applying to cell monolayers.

2. **Double Strength Agarose.** Double strength agarose (1.0 per cent), electrophoresis grade (General Biochemicals, Chagrin Falls, Ohio), was used for the initial overlay medium when human rhinovirus type 20 was titered by the plaque method. Agarose was used because it increased the size of the plaques.

3. **Magnesium Chloride (50X).** Magnesium chloride was used in the initial overlay medium (2 ml/100 ml) to give a 30 mM concentration of Mg$$^{++}$$ ions for enhancement of plaque formation. This 50X solution was prepared by dissolving 30.0 grams MgCl$_2$$\cdot$$6$H$_2$O in a final volume of 100 ml of distilled-demineralized water.

4. **Neutral Red.** A 0.1 per cent stock solution of neutral red (Hartman-Leddon Company, Philadelphia, Pennsylvania) in distilled-demineralized water was sterilized by autoclaving at 121° C for three minutes. Seven ml of this stock solution was mixed with 93 ml of the final overlay medium to give a 0.007 per cent final concentration of neutral red.
APPENDIX B

Viral Diluents

A. **Diluent for Plaque Assay of Viruses.** This diluent was prepared by adding the following ingredients to 696 ml of sterile distilled-demineralized water.

- Polymyxin B: 10,000 μg
- Streptomycin sulfate: 100,000 μg
- Potassium penicillin G: 100,000 units
- Earle's balanced salt solution, 10X without phenol red (GIBCO): 80 ml
- Sodium bicarbonate (7.5 per cent): 24 ml

B. **Diluent for TCID₅₀ Titration of Viruses.** This diluent was prepared by adding the following ingredients to 810 ml of sterile distilled-demineralized water.

- Chlortetracycline·HCl: 50,000 μg
- Polymyxin B: 10,000 μg
- Streptomycin sulfate: 100,000 μg
- Potassium penicillin G: 100,000 units
- Earle's balanced salt solution, 10X with phenol red (GIBCO): 100 ml
- Fetal bovine serum (Flow Lab., Inc.): 20 ml
- Tris base (1 M): 1.5 ml
Isotopes

The isotopes listed below were added to the culture medium (Appendix A) for incorporation into viral protein or nucleic acid. All isotopes were obtained from Schwarz Bioresearch, Inc., Orangeburg, New York. The individual amino acids and $^3\text{H}$-5-uridine were used at a concentration of 0.1 to 0.2 uc/ml of culture medium, while reconstituted $^3\text{H}$-protein hydrolysate was used at a concentration of 1.0 to 2.0 uc/ml of culture medium.

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Specific Activity</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^3\text{H}$-5-uridine</td>
<td>20.0 c/mmole</td>
<td>$^3\text{H}$-ur</td>
</tr>
<tr>
<td>$^3\text{H}$-L-leucine</td>
<td>2.0 c/mmole</td>
<td>$^3\text{H}$-leu</td>
</tr>
<tr>
<td>$^3\text{H}$-L-valine</td>
<td>0.6 c/mmole</td>
<td>$^3\text{H}$-val</td>
</tr>
<tr>
<td>$^{14}\text{C}$-L-leucine</td>
<td>338.0 mc/mmole</td>
<td>$^{14}\text{C}$-leu</td>
</tr>
<tr>
<td>Reconstituted $^3\text{H}$-protein hydrolysate</td>
<td></td>
<td>$^3\text{H}$-RPH</td>
</tr>
</tbody>
</table>
APPENDIX D

Buffers

A. Sodium Phosphate. One molar phosphate buffer pH 7.0 and 7.2 was prepared by dissolving 81.0 grams of Na$_2$HPO$_4$ and 59.3 grams of NaH$_2$PO$_4$·H$_2$O in a final volume of 1.0 liter of distilled water. This buffer was used for polyacrylamide gel electrophoresis and gradients when diluted appropriately. When prepared, the pH of this buffer was 6.8; it was adjusted to 7.0 and 7.2 with 10 N NaOH.

B. Trizma Base, Tris(hydroxymethyl)aminomethane (Tris). One molar Tris buffer was prepared by dissolving 12.11 grams of Trizma base (Sigma Chemical Company, St. Louis, Missouri) in a final volume of 100 ml of distilled water. This buffer was used unadjusted for cell culture media or diluted in distilled water to 0.01 M and adjusted with 10 N HCl to pH 8.1 for gradients.

C. Reticulocyte Standard Buffer (RSB). RSB was prepared by dissolving 1.211 grams Tris base, 0.7456 grams potassium chloride, and 0.30499 grams magnesium chloride in a final volume of 1.0 liter of distilled water. This buffer was adjusted to pH 7.6 with a 10 N HCl, and used as
diluent for sucrose gradients.

D. Tris-Na$_2$ ethylenediaminetetraacetate (EDTA) - Boric Acid Buffer (TEB). TEB buffer pH 8.0 was prepared by dissolving 43.1 grams Tris base, 2.20 grams boric acid, and 37 grams Na$_2$EDTA$\cdot$2H$_2$O in a final volume of 4.0 liters of glass distilled water. When prepared, the pH of this buffer was 8.1; it was adjusted to pH 8.0 with 10 N NaOH. This buffer was used as a diluent and electrode buffer for non-detergent PAGE.

E. Sodium Phosphate - Citric Acid Buffers. A 0.2 M stock solution of sodium phosphate and a 0.1 M stock solution of citric acid were prepared separately by dissolving 35.61 grams of Na$_2$HPO$_4$$\cdot$2H$_2$O and 21.01 grams of citric acid$\cdot$H$_2$O respectively in a final volume of 1.0 liter of distilled water. By mixing the appropriate volumes of each buffer, a citrate-phosphate buffer was obtained having a pH ranging from 3.0 to 8.0. These buffer combinations were used for inactivation studies of rhinoviruses.
APPENDIX E

Polyacrylamide Gel Electrophoresis (PAGE) Materials.

The structural components of human rhinovirus type 14 (HRV-14), before and after treatment at acid pH, were determined by PAGE. Electrophoresis was carried out in Buchler PAGE apparatus (Buchler Instruments, Inc., Fort Lee, New Jersey). The reagents for PAGE were: acrylamide, N, N'-methylenebisacrylamide, sodium dodecyl sulfate (SDS), N, N, N', N'-tetramethylethylenediamine (Temed), ammonium persulfate, 2-mercaptoethanol (2-ME), sodium phosphate, urea, trichloroacetic acid (TCA), Coomassie blue stain, acetic acid, and electrode buffer.

A. **Acrylamide** (Eastman Kodak Company, Rochester, New York, X 5521), 30 per cent w/v.

B. **N, N'-Methylenebisacrylamide** (Canal Industrial Corporation, Rockville, Maryland, No. 202), 0.8 per cent w/v.

C. **Sodium Dodecyl Sulfate** (SDS), 10 per cent w/v.
Ten grams of SDS (Schwarz/Mann, No. 1834) were dissolved and diluted to 100 ml with distilled water. SDS was used in PAGE buffer and to disrupt virus samples.

D. **N, N, N', N'-Tetramethylethylenediamine** (Temed).
Temed (Canal Industrial Corporation, No. 204) was used for initiating polymerization when ammonium persulfate was added to the acrylamide-bisacrylamide mixture.

E. Ammonium Persulfate (AP), 10 per cent w/v. Solid AP (0.5 g) Canal Industrial Corporation, No. 209) was dissolved in 4.5 ml of distilled water. This solution was made fresh each week and was stored under refrigeration.

F. 2-Mercaptoethanol (2-ME), 10 per cent v/v. A 10 per cent solution of 2-ME (Eastman Kodak Company, No. 4196) was prepared daily in 0.1 M sodium phosphate and 0.1 per cent SDS electrode buffer as diluent. It was used to reduce protein disulfide bonds.

G. Sodium Phosphate. (Appendix D) Sodium phosphate buffer was used in PAGE buffer.

H. Urea. Six M urea (Schwarz/Mann) was prepared by adding 0.036036 grams of solid urea/100 lambda of sample.

I. Trichloroacetic Acid (TCA). Three M TCA (Mallinckrodt Chemical Works, St. Louis, Missouri) was prepared by adding 9.25 ml of distilled water to a one pound bottle (453.6 grams) of TCA. This 3.0 M stock was used to make 0.6 M and 0.3 M TCA solutions.

J. Coomassie Blue Stain, 0.25 per cent w/v. This stain was prepared by dissolving 0.25 grams of Coomassie Brilliant Blue R-250 (Schwarz/Mann) in a final volume of 100 ml of distilled water. This stain was used to stain TCA fixed proteins following PAGE.
K. **Acetic Acid, 7.0 per cent v/v.** Acetic acid was prepared by diluting 70 ml of glacial acetic acid to a final volume of 1.0 liter with distilled water. It was used for destaining gels.

L. **Electrode Buffer.**

1. **Detergent.** An SDS buffer was prepared by adding 100 ml of 1.0 M sodium phosphate buffer (Appendix D) and 10 ml of 10 per cent SDS to 890 ml of distilled water. The pH was adjusted to 7.2 with 10 N NaOH.

2. **Non-detergent.** A non-detergent buffer containing Tris-Na$_2$ EDTA and boric acid (TEB) was prepared as listed in Appendix D.
APPENDIX F

Estimation of Molecular Weights by Internal Standards

Polyacrylamide gel electrophoresis (PAGE) provides a method for the analytical or preparative separation and collection of polypeptides, proteins, nucleic acids, and other macromolecules that range in molecular weight from millions of daltons to several hundred daltons. Probably the most common use of PAGE is to quantitate not only the number of polypeptides or proteins present in a particular sample, but also the size in daltons for a particular protein or polypeptide. In order to estimate the molecular weight of an unknown protein, standard calibration curves must be constructed for every gel concentration that is used. With the versatility of being able to select a wide range of gel concentrations and pore size, the requirement for more than one standard curve becomes obvious. The most popular and general method for estimating molecular weights in an SDS-PAGE system is by using external standards to construct a standard calibration curve. Generally, a minimum of at least five known standards are used to make a standard curve.

The use of internal standards allows for a rapid and accurate method for estimating the molecular weight of
an unknown component in an SDS-PAGE system. By using internal standards relative mobilities need not be determined. Also, experimental variations induced from gel to gel can be reduced by co-electrophoresing standards and unknowns all within the same gel. The method described herein intends to offer a rapid and general procedure for estimating unknown molecular weights by using internal standards.

The relationship between the molecular weight of a protein or polypeptide and its migration in an SDS-PAGE system has been documented (95). This relationship can be expressed as:

\[
(1) \quad \text{Molecular weight } (Y) = be^{-kx}
\]

where \(k\) and \(b\) represent the constants of slope and \(Y\) intercept respectively, and \(x\) the distance of migration or relative mobility of a particular protein or polypeptide. If the logarithm of both sides of equation 1 is taken, a linear equation for \(x\) and \(\ln b\) results where \(k\), \(x\), and \(\ln b\) correspond to the slope, migration, and \(Y\) intercept respectively. Theoretically, only two known molecular weight standards are necessary for the estimation of the molecular weight of any unknown protein or polypeptide. For this to be true, both known and unknown samples must be run in the same gel. By using a minimum of only two known standards in a gel, equation 1 can be solved for \(b\) and \(k\) by using the
following relationship.

\[ Y_1 = \frac{b e^{-kx_1}}{Y_2} \]

\[ k = \ln \frac{Y_1}{Y_2} \cdot \frac{1}{x_2-x_1} \]

\[ b = \frac{Y}{e^{-kx}} \]

After \( k \) has been determined, the molecular weight of any unknown in the same gel can thus be determined by the following relationship.

\[ Y(\text{unknown}) = Y(\text{known}) \cdot e^{k(x_2 - x_1)} \]

Using the above relationship, the molecular weights of poliovirus and rhinovirus VP 1, 2, and 3 were estimated. Estimation was accomplished by using any two virus VP's as knowns to solve for the third virus VP. The fast migrating virus polypeptide (VP 4) was not estimated for each virus since it never migrated as a sharp peak, and its specific direct mobility could not be accurately determined. The results are given in Table 11 and show that the apparent estimated molecular weights do not deviate by more than 20 percent from the molecular weights reported in the literature.

It has been shown that internal standards can be used to determine the molecular weight of unknown components in polyacrylamide gels. To obtain a better estimation
# TABLE 11

**ESTIMATION OF MOLECULAR WEIGHTS BY INTERNAL STANDARDS**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Molecular Weight</th>
<th>Migration mm</th>
<th>Apparent Molecular Weight</th>
<th>Per cent Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poliovirus Type 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP 1</td>
<td>32,000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21</td>
<td>26,600</td>
<td>16.8</td>
</tr>
<tr>
<td></td>
<td>32,000</td>
<td>22</td>
<td>26,000</td>
<td>18.8</td>
</tr>
<tr>
<td>VP 2</td>
<td>25,000</td>
<td>24</td>
<td>27,300</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>25,000</td>
<td>24</td>
<td>27,300</td>
<td>9.2</td>
</tr>
<tr>
<td>VP 3</td>
<td>24,000</td>
<td>26</td>
<td>20,500</td>
<td>14.6</td>
</tr>
<tr>
<td></td>
<td>23,000</td>
<td>26</td>
<td>20,500</td>
<td>14.6</td>
</tr>
<tr>
<td>HRV-14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP 1</td>
<td>42,000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55</td>
<td>40,900</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>42,000</td>
<td>58</td>
<td>40,600</td>
<td>3.3</td>
</tr>
<tr>
<td>VP 2</td>
<td>33,000</td>
<td>64</td>
<td>33,300</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>33,000</td>
<td>68</td>
<td>33,100</td>
<td>0.3</td>
</tr>
<tr>
<td>VP 3</td>
<td>30,000</td>
<td>68</td>
<td>29,700</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>30,000</td>
<td>72</td>
<td>30,000</td>
<td>0.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Molecular weights of poliovirus type 2 polypeptides as reported by Holland, J. J. and Kiehn, E. D. (46).

<sup>b</sup> Molecular weights of rhinovirus type 14 polypeptides as reported by Korant et al. (59).

<sup>c</sup> Per cent deviation determined by: molecular weight minus apparent molecular weight, divided by molecular weight times 100.
of an unknown molecular weight, it is better not to use two standards having the same order of magnitude. Since the relationship between migration and molecular weight follows an exponential law, the use of two standards of similar magnitudes may lead to errors on each end of the standard curve. This is especially true when the standard curve is extrapolated away from the standards. If standards having a similar order of magnitude are used, estimations of unknown molecular weights should be limited to regions just around the standards. The error of molecular weight estimation by using internal standards can be reduced by:

(1) using standards having a different order of magnitude, or
(2) by using more than two internal standards.
BIBLIOGRAPHY


10. Burroughs, J. M., Rowlands, D. J., Sangar, D. V.,
    Talbot, P., and Brown, F. (1971). Further
    evidence for multiple proteins in the foot-and-
    13:73.

    Inoculation of human volunteers with a strain
    of virus isolated from a common cold. Lancet
    1:1194.

12. Gate, T. R., Douglas, R. G., Johnson, K. M., Couch,
    R. B., and Knight, V. (1967). Studies on the
    inability of rhinoviruses to survive and
    replicate in the intestinal tract of volunteers.

    studies of a rhinovirus: Ultracentrifugation

    properties of picornaviruses: A comparative
    study of human and animal enteroviruses and
    rhinoviruses. Ph.D. dissertation, The Ohio
    State University.


    of the numbering system. Virol. 43:524.

17. Conant, R. M. and Hamparian, V. V. (1968). Rhino-
    viruses: Basis for a numbering system. I.
    HeLa cells for propagation and serologic

18. Conant, R. M. and Hamparian, V. V. (1968). Rhino-
    viruses: Basis for a numbering system. II.
    Serologic characterization of prototype-strains.

19. Conant, R. M., Somerson, N. L., and Hamparian, V. V.


40. Hamparian, V. V. Personal communication.


