ing that glucose phosphorylation was not a factor in uptake inhibition.

Discussion. Previously it was shown that the rates of growth and glucose catabolism of yeast were affected immediately when cells were transferred from H_2O to D_2O or from D_2O to H_2O, whereas the half time for equilibration was at least 10 minutes(1). From the results presented here, it is possible to account for the rapid response to changing the isotopic composition of the suspending medium. The glucose, and to a lesser extent, phosphate uptake processes were shown to be sensitive to D_2O inhibition independent of metabolic effects. Since transport systems are located on or near the cell surface, uptake would be affected almost immediately when cells are transferred to D_2O or H_2O, resulting in an abrupt change in the rates at which glucose and phosphate enter the cell. This, in turn, would cause a rapid alteration in the rates of both growth and glucose catabolism. In addition to affecting rates of growth and metabolic activity, it is likely that uptake inhibition would cause physiological imbalances within the cell and thus lead to further metabolic disturbances.

Summary. Deuterium inhibited uptake of glucose, potassium, and phosphate. Potassium uptake inhibition was probably due to the depressed rate of glucose catabolism in D_2O. Glucose and phosphate uptake inhibition was due to direct isotope effects on the transport processes. Uptake and the effects of D_2O on uptake were the same for deuterio-glucose as for ordinary glucose. Results of experiments on the effects of D_2O on sorbose and glucose transport indicated that these sugars enter the cell by different mechanisms. It was concluded that the immediate effects of D_2O in depressing the rates of growth and glucose catabolism were due to inhibition of glucose and phosphate uptake.

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Identification of the Antiviral Substances in Nasal Secretions. (29637)

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Over the years, investigations of local immunity of the respiratory tract have led to a search for antibodies or antibody-like activity in respiratory secretions. Substances capable of inhibiting proliferation of viruses have been described repeatedly in respiratory mucus(1) although the nature of the inhibiting substances has not been clearly elucidated. Primarily on the basis of experiments showing very little anti-influenzal activity when incubation of nasal secretions and virus was carried out at 4°C, Burnet *et al*(2) suggested that the virus inactivating substance was different from serum antibody. Francis studied nasal antibodies to influenza in humans and found a positive correlation with serum titer, and certain biochemical and serologic characteristics which suggested the active material to be similar to serum antibody(3). Fazekas St. Groth found that influenzal immunization of mice produced a parallel response of serum and nasal antibodies(4). He also demon-

strated transfer of circulating antibody into bronchial washings of mice following intraperitoneal inoculation with immune serum (5). These results suggested a serum origin of respiratory antibodies.

The present studies were undertaken to investigate the capacity of human nasal secretions to neutralize a number of different viruses and to attempt identification of the neutralizing substance. Evidence will be presented that the neutralizing activity is associated with γ1A-globulins in these secretions.

Materials and methods. Subjects. Ten adult male laboratory workers contributed normal secretions. Three other men and one of the normals were studied during periods of respiratory disease. Three patients with chronic bronchitis, in remission, were also tested.

Collection and processing of nasal secretions. In preliminary trials, the use of cotton plugs, stimulation of nasal mucosa with sodium carbonate and washing of nasal cavities with distilled water were found to be less satisfactory for collection of nasal secretions than saline washes. The following technique was used throughout these studies. With neck hyper-extended and glottis closed, 5 ml of sterile 0.15 M saline solution was instilled into one nostril by pipette. The solution was then forcibly expelled through the nares into a clean beaker. Washings were collected several times daily for several days; the secretions from each man were then pooled and frozen at —20°C. Nasal washings were then prepared for assay of antiviral activity. After thawing, the specimen was shaken vigorously with glass beads, centrifuged at 500 *g* for 20 minutes, dialyzed overnight against distilled water, and concentrated by lyophilization. After rehydration with distilled water to one-tenth the initial volume, total protein was determined by the sulfosalicylic acid method (6). Specimens were adjusted to a final protein concentration of 150 mg% ± 15 mg%. All samples were found to be free of hemoglobin by the guaiac test.

Cell cultures and viruses.* Rhesus monkey kidney cell cultures were used for tests with influenza, parainfluenza, polio and Coxsackie viruses. HEP_2 cultures were used for adenovirus neutralization tests; diploid human embryonic lung cultures were used for ECHO 28 and rhinovirus antibody determinations. The viruses were standard strains used in this laboratory, each with multiple cell culture passages. Influenza A_2/Japan/170/62 was obtained from Dr. J. A. Morris as allantoic fluid and was adapted to tissue culture; ECHO 28 and rhinovirus 1059 were obtained from Dr. M. Mufson.

Technique of neutralizing tests. Preliminary studies had indicated that concentrated fresh nasal washings were invariably contaminated with fungi or bacteria. Heating at 56°C for 30 minutes sterilized them and comparative titrations showed no loss of virus neutralizing capacity upon heating. Heat inactivated concentrated nasal secretions, or serial 2-fold dilutions of serum were mixed with an equal volume of virus fluid containing 30 to 300 tissue culture infective doses (TCID 50). Following 1 to 3 hours of incubation at room temperature, 2 culture tubes were inoculated with 0.1 ml of each mixture. Tests were read 2 to 5 days later. Hemadsorption served as the indicator for myxoviruses; cytopathic effect for the other agents.

Immunoelectrophoresis of nasal secretions and sera. The method of Grabar and Williams(7) as adapted to microscope slides by Scheidegger(8) with minor modifications was used. Horse anti-human serum (AHS) was obtained from Dr. Elmer L. Becker. Goat anti-human γ1A-globulin antiserum (Lot G206, Hyland Laboratories, Los Angeles, Calif.) produced a single line in immunoelectrophoresis against normal undiluted human serum. (In one preparation in which serum (human) was diluted 1:20 a faint alpha-globulin arc was noted in addition to the γ1A-globulin line.) Goat anti-human γ2-globulin antiserum (lot GP 11-63, Hyland Laboratories) produced a single line in Ouchterlony plates when tested with undiluted normal human serum, but by immunoelectrophoresis against serum diluted 1:20, a cross reaction with γ1A-globulin was observed.

* Purchased from Microbiological Associates, Inc., Bethesda, Md., and Flow Laboratories, Rockville, Md.

TABLE I. Activity and Nature of Antibody in Normal Nasal Secretions.

	Neutralizing activity to indicated viruses								Immunoelectrophoresis			
										Globulins		
Patient	Influenza A_2/62	Influenza A_1	Para-influenza Type 3	Polio Type 1	Adeno-virus Type 7	Coxsackie A9	ECHO 28	Rhino 1059	Albumin	Alpha	γ1A	γ2
1	0	+	+	0	0	0	0	0	+	±	+	0
2	+	+	+	+	0	+	+	0	+	+	+	0
3	+	+	+	0	0	+	0	0	+	+	+	0
4	0	0	+	+	0	0	+	0	+	+	+	0
5	+	0	0	+	0	0	+	0	+	+	+	0
6	+	+	+	0	+	0	+	+	+	+	+	0
7	+	+	+	+	0	0	0	ND	+	+	+	0
8	+	+	+	+	+	+	+	0	+	±	+	±
9	+	+	+	0	0	0	+	+	±	+	+	0
10	0	+	+	+	0	+	0	+	±	+	+	0

+ = present; 0 = not present; ND = not done; ± = trace amount.

TABLE II. Activity and Nature of Antibody in Nasal Secretions from Patients with Respiratory Diseases.

		Neutralizing activity to indicated viruses								Immunoelectrophoresis			
											Globulins		
Diagnosis	Patient	Influenza A_2/62	Influenza A_1	Para-influenza Type 3	Polio Type 1	Adeno-virus Type 7	Coxsackie A9	ECHO 28	Rhino 1059	Albumin	Alpha	γ1A	γ2
Cold	1A*	+	+	+	+	0	0	+	0	+	0	±	±
"	1B	+	+	+	+	0	0	+	0	+	0	±	+
Hay fever	2	ND	+	+	+	+	0	ND	ND	+	+	+	0
Allergic rhinitis	3	+	+	+	+	0	0	0	0	+	+	+	+
Pharyngitis	4	+	+	+	+	0	0	+	0	+	+	+	±
Bronchitis	5	0	+	+	+	0	+	0	+	±	+	+	0
"	6	+	+	+	0	0	0	0	0	+	+	+	0
"	7	+	+	+	+	+	0	+	+	+	+	+	0
"	5†	0	+	+	+	0	+	0	ND	+	+	+	0
"	6†	+	+	+	0	0	0	0	ND	+	+	+	0
"	7†	+	+	+	+	+	0	+	ND	+	+	+	0

* Patient 1, same individual as normal patient 1 (Table I). Specimen A taken 12/11/62. Specimen B taken 2/2/63. † Sputum specimen.

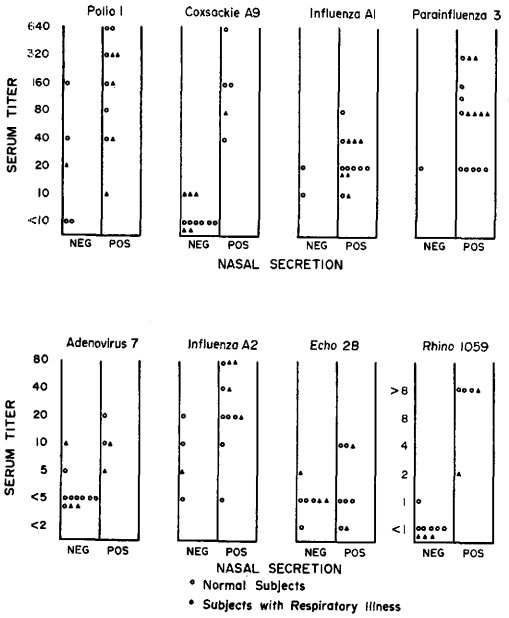

FIG. 1. Relation of nasal antibody to serum neutralizing titer.

Results. Neutralization of viruses by nasal secretions. Neutralizing activity against 2 or more of the 8 viruses tested was found in each of the 10 normal nasal washings (Table I). That this activity was specific was suggested by the fact that no 2 specimens neutralized the same viruses and that the activity against closely related viruses varied; for instance, nasal secretions of 3 subjects (#1, 5 and 10) reacted differently to influenza A_1 and A_2 viruses. In addition, neutralization of a number of different animal viruses was observed; *e.g.*, myxoviruses, enteroviruses, rhinoviruses and an adenovirus were neutralized by 2 or more secretions. Seven persons with respiratory illness also showed antiviral activity in nasal secretions or sputum (Table II).

The relationships between serum neutralizing antibody titer and nasal antibody, tested simultaneously, are depicted in Fig. 1. With few exceptions a high serum titer was associated with virus inactivation by nasal secretion; absence of serum antibody was associated with lack of nasal antibody in most cases. However, 5 patients with undetectable serum neutralizing antibody to ECHO 28 virus ($<1:2$ or $<1:5$), and one patient with no demonstrable influenza $A_2/62$ antibody in serum ($<1:5$), were found to have antiviral activity in nasal secretions. No significant differences between normal and diseased persons were observed.

Immunoelectrophoresis of secretions. Properties of the virus neutralizing substance in normal nasal secretions suggested it to be protein. Thus, activity was retained following heating at 56°C for 30 minutes, freezing, dialysis and lyophilization and was destroyed by boiling or trypsin digestion for one hour. Secretions were characterized further by immunoelectrophoresis. In Table I are listed the protein components found in normal nasal secretions demonstrating specific antiviral activity. All specimens contained albumin, alpha and γ1A-globulins. One specimen (Subject #8) contained trace amounts of γ2-globulin. It is of interest that γ2-globulins were rarely found in nasal secretions from normal persons and when present could be identified by the present techniques only in trace amounts.

Similar studies with secretions obtained from persons with respiratory illness are indicated in Table II. Albumin was found in all of these specimens but the alpha globulin precipitin arc was absent from specimens 1A and 1B. Gamma 1A-globulin was found in all cases; γ2-globulins were noted in secretions from 4 patients with rhinitis (#1A, 1B, 3 and 4). Purulent nasal secretions from other patients with acute respiratory disease, not shown in the Tables, routinely contained γ2-globulins, and often several β-globulins were identified. Fig. 2 illustrates the immunoelectrophoretic appearance of nasal secretions from normals and an individual with respiratory disease.

The beta-globulin of normal secretions was identified as a γ1A by its typical immunoelectrophoretic migration. This was confirmed by precipitation with specific goat anti-human γ1A antiserum. By the Ouchterlony double diffusion system (Fig. 2D) a reaction of identity between serum γ1A-globulin and the γ1A-globulin of normal nasal secretions was observed.

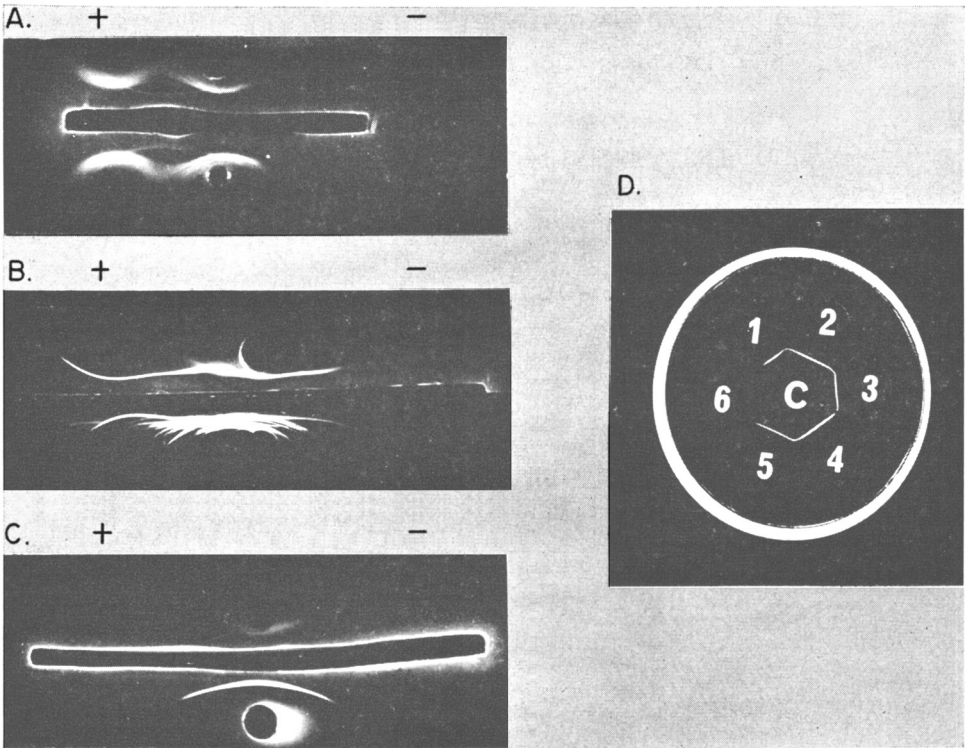

FIG. 2. Immunoelectrophoresis of nasal secretions. A. Normal nasal secretions in both antigen wells; trough contains horse anti-whole human antiserum (AHS). B. A composite figure showing in upper well nasal secretion obtained during respiratory infection; lower well has a normal human serum; trough contains AHS. C. Upper well contains normal nasal secretion, diluted 1:2 with saline; bottom well contains undiluted normal serum. Trough is filled with goat anti-human γ1A-globulin antiserum. D. Ouchterlony plate in which well 1 holds sputum, wells 2 and 4 contain normal human serum, wells 3 and 5 contain normal nasal secretions, well 6 contains human γ2-globulin, and center well is filled with specific γ1A antiserum.

Absorptions to remove immunoglobulins. Since normal nasal secretions contained γ1A-globulins predominantly and γ2-globulins infrequently, it seemed likely that the antiviral substance in these secretions was related to their γ1A-globulin content. A number of experiments were performed in which the γ1A-globulin was removed by absorption of the secretions with specific γ1A antiserum.

Equal volumes of secretion and antiserum were incubated 24-48 hours at 4°C; precipitates were removed by centrifugation (500 *g* for 20 minutes). An aliquot of secretion mixed with an equal volume of 0.15 M saline was treated similarly. Since the goat antiserum was toxic to rhesus kidney cell cultures, the neutralization tests were modified by washing the serum-virus mixture from each tissue culture tube after allowing one hour for free virus to adsorb to the monolayer.

Following absorption of normal nasal secretions or sputum with γ1A antiserum, all detectable neutralizing activity against parainfluenza type 3, Echo 28, Coxsackie A9, polio type 1 and influenza A2 viruses was removed (Table III). However, influenza antibody was not removed from nasal secretions of 2 patients (#5 and 6) with acute respiratory disease. Treatment of these 2 specimens with γ2 antiserum resulted in loss of anti-influenza activity. Absorption of a normal nasal secretion (#2) with γ2 antiserum resulted in complete removal of the trace amount of γ2-globulin present and partial removal of the γ1A-globulin. There as no loss of Echo 28 antibody but there was a significant reduction of Coxsackie A9 and

TABLE III. Removal of Immunoglobulins and Antiviral Activity of Respiratory Secretions by Absorption.

Patient	Type of specimen	Virus tested	Control		γ1A absorbed		γ2 absorbed	
			N* titer	Globulin content	N titer	Globulin content	N titer	Globulin content
1	Nasal, normal	Parainfluenza 3	1:1–1:2	γ1A, γ2	<1:1	γ2	ND‡	ND
		ECHO 28	1:16	"	<1:1	"	ND	ND
2	*Idem*	*Idem*	1:16	γ1A, γ2±	<1:1	γ2±	1:16	γ1A±
		Coxsackie A9	1:4	"	<1:1	"	<1:1	"
		Polio type 1	1:16	"	<1:2	"	1:2	"
3	Sputum, bronchitis	Parainfluenza 3	1:2	γ1A	<1:1	0	ND	ND
4	*Idem*	Influenza A_2	1:16	γ1A	<1:1	0	ND	ND
5	Nasal, ARD†	*Idem*	1:4	γ1A, γ2	1:4–1:8	γ2	<1:1	γ1A
6	*Idem*	"	1:4–1:8	ND	1:4–1:8	ND	<1:1	ND

* N = Neutralizing antibody. † ARD = Acute respiratory disease. ‡ ND = Not done.
± = Trace amount.

polio 1 antibody. That this was not due to incomplete precipitation of high titered antibody is shown by the fact that absorption removed 8 or more units of polio 1 and Coxsackie A9 antibody but none of comparably titered antibody to Echo 28 virus. This suggests that the antibody to Echo 28 virus was composed primarily of γ1A-globulins, whereas that to polio 1 and Coxsackie A9 was a mixture of γ1A- and γ2-globulins. An alternate explanation for the patterns of absorption for subject #2 is that absorption with γ2 antiserum selectively removed the polio 1 and Coxsackie A9 but not the Echo 28 antibody. Although additional absorptions with γ2 antisera were not performed this explanation hardly seems likely. Finally, immunoelectrophoretic analysis of the γ1A and γ2 antisera showed good specificity of the former and partial cross reaction of the latter.

Discussion. The present findings suggest that the antiviral substance of normal nasal secretions is predominantly γ1A-globulin, closely related or identical to serum γ1A-globulin. During acute inflammatory conditions γ2-globulin is almost always present in nasal secretions and in this situation it appears to be the predominant antiviral antibody. Because of the specificity of the antiviral activity in individual secretions and its removal by anti-globulin absorptions the active substance does not appear to be an interferon such as that described in throat washings in acute influenza(9). Although albumin and an alpha globulin have been detected in almost all respiratory secretions tested these have not been associated with the antibody activity in subsequent studies in this laboratory using gel filtration separation(10).

The presence of numerous serum proteins in nasal secretions obtained during respiratory infection has been previously described by Anderson, Riff and Jackson(11). Their failure to detect γ1A-globulins in normal nasal secretions may reflect the use of a less potent anti-human antiserum (AHS) than was used in the current studies. The horse AHS used in their studies detected 12 to 14 distinct antigenic components in whole human serum; the horse AHS used in the present studies detected over 25 components.

Remington and Vosti(12) have also described the occurrence of albumin and β2A (γ1A) globulins in normal nasal secretions possessing hemagglutinins for tetanus toxoid and streptococcal M protein. The precise identification of the active component was not established. In a study of normal human tracheobronchial washings, Keimowitz found high concentrations of β2A (γ1A)-globulins in comparison to albumin and γ2-globulin (13). Together, while not defining the activity of various globulin components, these observations support the present studies by independent confirmation of their presence in respiratory secretions.

The mechanism by which the γ1A-globulins appear in normal respiratory secretions remains to be established. The quantitative studies of Chodirker and Tomasi(14) have

demonstrated that γ1A-globulin is present normally in several body secretions in relatively greater concentration than in serum. These authors have postulated a selective process of concentration and transport of γ1A-globulins from the serum into these fluids. If this is indeed the case, the antibody in normal respiratory secretions may be derived directly from humoral antibody. Evidence has been presented recently, however, that the γ1A-globulin found in local secretions may differ in antigenic characteristics from the γ1A-globulin of serum(15). This would suggest that modification of the γ1A-molecule occurs during transport to the local site or, that a change in the molecule is produced by local enzymes, or that local production of γ1A-globulins occurs.

Finally, it is known that in allergic individuals certain γ1A-globulins behave as reagins (skin sensitizing activity) in both serum (16) and nasal secretions(12). Whether, in combining with viruses *in vivo* γ1A-globulins from normal persons behave similarly is unknown. However, if they do possess reaginic activity, it is tempting to speculate that this reaction may contribute to the altered vascular permeability characteristic of respiratory infections. Further studies of this problem and that of the relation of the antibody in nasal secretions to immunity are currently in progress.

Summary. Antiviral activity of nasal secretions was studied using 8 different viral agents and secretions collected from 10 normal adults and 7 persons with acute respiratory symptoms. Neutralizing activity of nasal secretions correlated well with the presence of serum antibody to each virus tested. Immunoelectrophoretic preparations and immunoglobulin absorptions revealed γ1A-globulin to be the predominant immunoglobulin found in normal nasal secretions although trace amounts of γ2-globulin were occasionally found. In secretions from normals, antiviral activity was primarily associated with the γ1A-globulins. During acute respiratory infections a larger number of serum proteins were detected in nasal secretions and antiviral activity was predominantly of the γ2-globulin variety.

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