carbohydrate metabolism which accompany rapid tissue growth and that coenzyme A may not play an important role in protein synthesis. 2. An unexplained rise in renal coenzyme A concentration was observed 24 hours after partial hepatectomy.

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Enzymic Action of Influenza Virus on Human Erythrocyte Stroma Components.* (23401)

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Sialic acid, a compound originally isolated from bovine submaxillary gland by Blix(1) is now recognized as an important constituent of soluble inhibitors of influenza virus hemagglutination. Its structure, proposed by Gottschalk(2), has only recently been established by the work of Heimer and Meyer(3). This chromogenic substance has also been identified among the dialyzable split products resulting from the action of influenza virus on certain mucoids(4). Interest in sialic acid in relation to action of influenza virus has led to a reexamination of receptor substances in human erythrocytes. The present study is based on earlier work(5) in which it was shown that virus receptors and blood group antigens could be concentrated from lyophilized erythrocyte stroma by relatively simple ultracentrifugation methods into a lipid-rich carbohydrate-containing fraction. This fraction has now been found also to contain a measurable proportion of chromogenic material giving absorption spectra in the direct

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Ehrlich and Bial reactions identical with those of crystalline sialic acid and certain soluble inhibitory mucoids. The erythrocyte chromogen has been recognized among the dialyzable split products resulting from the action of infective influenza virus on erythrocyte stroma and purified receptor substance derived therefrom(6).

Materials and methods. Influenza viruses were propagated by chorioallantoic inoculation of embryonated eggs 11 days of age, the fluid being harvested after 48 hours of incubation at 37°C.‡ Indicator strains were made by heating infective chorioallantoic fluid to 56°C for 2 hours. Hemagglutination inhibition tests were performed at room temperature, 5 hemagglutinating units for 0.1 ml of 2% chicken erythrocytes being the standard amount of virus in a final volume of 0.7 ml. End points were read by the pattern technic after approximately one hour. 0.9% sodium chloride, buffered to pH 7.2 with one part in 10 of M/15 phosphate buffer, was used as diluent. Nitrogen was measured by the Markham modification of the microKjeldahl procedure(7). Hexosamine was determined on hydrolyzed samples by the method of Elson and Morgan(8) and reducing sugar by that of Hagedorn and Jensen(9). Total phosphorus was determined by the method of King (10). Sialic acid was estimated by the direct Ehrlich and the Bial (orcinol) color reactions as described by Werner and Odin(11). Absorption at 560-565 in the direct Ehrlich reaction and that at 570 in the Bial reaction were used as the basis for calculating sialic acid content. Since the Ehrlich chromogen tended to fade rapidly, all readings were taken within 15 minutes of cooling. Crystalline sialic acid from bovine submaxillary mucoid(3)§ was used as a reference standard. Two soluble inhibitors of viral hemagglutination were also used as reference material. The first, human brain mucolipid (BML), was generously supplied by Dr. A. Rosenberg, and had been prepared by him according to methods already described(12). Human brain mucolipid contained 14-16% sialic acid and a minimum of 12-50 μg inhibited indicator PR8 and PR301. The second, sputum inhibitor (SpI), was prepared by Dr. B. F. Erlanger by the procedure of Marmion, *et al.*(13). It contained 4% sialic acid and a minimum of 2-10 μg inhibited several indicator strains. Total solids in dialyzed concentrated stroma were determined by drying measured samples to constant weight *in vacuo* over phosphorus pentoxide. Calcium borate buffered saline at pH 7.1-7.3 was prepared according to the formula of Burnet and Stone(14).

Preparation of stroma fraction containing virus receptor substance. Fresh human erythrocytes were washed 3 times in 0.9% sodium chloride solution, and hemolyzed with 9 volumes of cold distilled water. The precipitate obtained after adjustment of the hemolyzate to pH 5.5 with acetate was collected by centrifugation and brought to pH 7.4 and lyophilized. The lyophilized material was then redissolved in water at pH 7.4-8.0, brought to sodium chloride concentration of 0.15 M, and centrifuged in a model L Spinco ultracentrifuge, using either a 40 head (80,000 G) or a 21 head (40,000 G). The sediment was resuspended in 0.15 M saline, and recentrifuged 3 more times under the same conditions, the supernatant fluids being discarded. The last sediment (P4) was resuspended in a small volume of water and dialyzed against water before analysis. Certain preparations were then also dialyzed against calcium borate buffered saline for use in virus enzyme experiments. Analytical values for 4 preparations of P4 are given in Table I. The nitrogen concentration, taken as protein nitrogen, accounts for up to half the total weight. Values for reducing sugar and hexosamine are probably minimal, since conditions of hydrolysis other than those stated were not investigated. Phosphorus was measured in 3 preparations, and is present probably as phospholipid, which therefore would account for 27-30% of the total weight, using the usual factor of 25. The spectra of both the direct Ehrlich and the Bial chromogens corresponded precisely with those for sialic acid as shown in Fig. 1 and 2. All preparations gave a straw-colored opalescent solution in water,

‡ The authors are indebted to Dr. Alice W. Knox for preparation of influenza virus.

§ Kindly supplied by Dr. R. Heimer.

TABLE I. Erythrocyte Stroma Fraction (P4) Containing Virus Receptor Substance.

Preparation No.	Donor	% nitrogen	% reducing sugar (as glucose) †	% hexosamine†	% chromogen Direct Ehrlich	% chromogen Bial	% phosphorus
1	Sch	8.0	4.5	1.8	2	1	1.2
2	Laz	7.5	4.5	2.1	2	2	1.1
3	Fri	6.8	4.8	1.8	2	2	1.2
4*	A	4.7	2.4	.9	n.d.	1	n.d.

* Preparation 6 in Table II.
† After hydrolysis (2N HCl, 2 hr) and neutralization.

and inhibited indicator virus hemagglutination in amounts of 25-100 μg.

Liberation of chromogen from stroma concentrate (P4) by concentrated influenza virus. Freshly harvested infective chorioallantoic fluid was centrifuged at 25,000 rpm (41,000 G) for 60-90 minutes, a 10- to 15-fold concentration of hemagglutinin being achieved by this procedure. The virus pellet was taken up in calcium borate buffered saline. Samples of P4 in amounts of approximately 0.5 g, following dialysis against calcium borate buffered saline or water, were then mixed with 70-140 μg total viral nitrogen. Appropriate controls were included, namely stroma with heat-inactivated virus (70° 10 minutes) or without virus, and active virus alone. Following incubation at 37°C for 16-18 hours, all samples were dialyzed against water and the dialyzates analyzed for chromogen by either direct Ehrlich or Bial reactions or both. The nondialyzable residues were heated to 70°C for 10 minutes to inactivate residual virus, and tested for hemagglutination inhibition with indicator strains. In the upper part of Table II, the results are given of a number of experiments in which 6 different preparations of P4 were treated with 5 different strains of active influenza virus. Loss of inhibitory activity, as

TABLE II. Effect of Concentrated Active Influenza Virus on Various Inhibitors of Hemagglutination.

Preparation	Strain of concentrated active virus	Chromogen liberated (as % of total) Direct† Ehrlich	Chromogen liberated (as % of total) Bial	Loss of inhibitory activity for indicator PR8	PR301	FM1	Lee	GL
Erythrocyte stroma (P4)								
1	PR8	31	31	+	+	+	+	+
	PR301‡	33		+	+	—		
2	PR301‡	40		+	+	—		
3	PR301		31	+	+	—	+	+
No. 3 after treatment with PR301	FM1		26			+		
3	FM1		46	+	+	+	+	+
4	FM1		27	+	+	+	+	+
5	Lee	18	22	+	+	+	+	+
6*	GL		37	+	+	+	+	+
Erythrocyte stroma inhibitor (ESI)†								
1	PR301§		27	+	+	—	+	+
2	FM1		56	+	+	+	+	+
Sputum inhibitor (SpI)	PR8	26; 35‖		+		+	+	+
	PR301‡	31	36	+		+	+	+
Human brain mucolipid (BML)	PR8	26; 27¶		+	+			
	PR301§		15	+	+			

* Preparation 4 in Table I. † Correspond with preparations 1 and 2, Table III. ‡ Same exp. § Same exp. ‖ 2 separate exp. ¶ Two separate exp.

indicated by a +, regularly represented a 50-fold or greater difference in inhibition titer between the material treated with active virus and the corresponding control, and frequently complete absence of inhibitor in the virus-treated sample. The controls regularly retained a full titer of inhibitor. In all instances, inactivation of the substrate was accompanied by the liberation of appreciable chromogen in dialyzable form, these amounts being expressed as a fraction of the total chromogen originally present in the reaction mixture. Control dialyzates consistently failed to give any color reaction. For purposes of comparison, sputum inhibitor (SpI) and brain mucolipid (BML) were included in experiments with PR301 and PR8. The results indicate that with these soluble materials as well destruction of or marked reduction in inhibitory activity as a result of viral action was accompanied by release of appreciable dialyzable chromogen. It is of interest that in 3 separate experiments with 3 different preparations of stroma P4, PR301 regularly failed to inactivate receptors for FM1; and in one experiment (preparation 3) subsequent treatment with FM1 of the substrate already treated with PR301 resulted in inactivation of FM1 receptors as well as in liberation of still more chromogen, *i.e.* 26% of what was left after PR301 treatment. Fig. 3 gives the absorption spectra of the dialyzable chromogen obtained with PR301 from erythrocyte stroma, sputum inhibitor, and brain mucolipid, with both the Bial and the direct Ehrlich reactions. With the exception of the dialyzable chromogen from preparation ES (Sch) P4, which in the Bial reaction gave a maximum slightly lower than 570, the curves correspond closely with those for sialic acid and the parent stroma fraction P4 as shown in Figs. 1 and 2.

Isolation of soluble erythrocyte stroma inhibitor (ESI). Since the relatively crude erythrocyte stroma concentrate (P4) described above contained appreciable lipid, the partition dialysis technic of Folch(15) was applied in a manner similar to that used by Rosenberg, *et al.*(12) for the isolation of brain mucolipid. In a typical preparation, approximately 2 g of P4 in the form of a

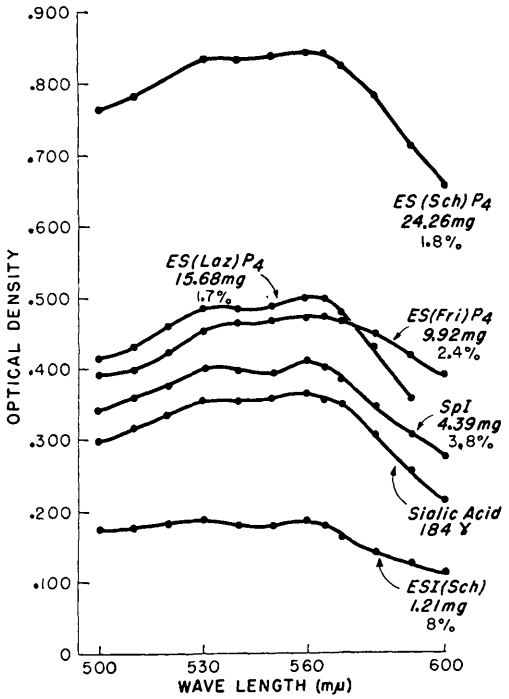

FIG. 1. Absorption spectra of chromogen (direct Ehrlich) in erythrocyte stroma concentrate (ES P4), erythrocyte stroma inhibitor (ESI) and sputum inhibitor (SpI), compared with spectrum of crystalline sialic acid.

thick paste were homogenized at 0°C for 2-3 minutes in a Waring blendor with about 400 ml chloroform-methanol (2:1, v:v). The whole mixture was then filtered, and the fil-

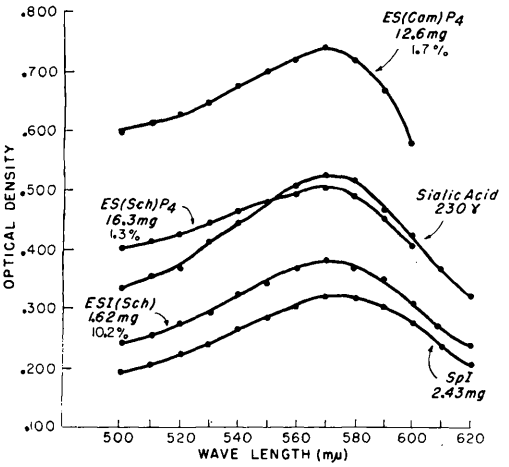

FIG. 2. Absorption spectra of chromogen (Bial) in erythrocyte stroma concentrate (ES P4), erythrocyte stroma inhibitor (ESI) and sputum inhibitor (SpI), compared with spectrum of crystalline sialic acid.

TABLE III. Erythrocyte Stroma Inhibitor (ESI).

Preparation*	% nitrogen	% reducing sugar (as glucose)†	% hexosamine†	% chromogen Direct Ehrlich	% chromogen Bial	% phosphorus	μg inhibiting 5 hemagglutinating units indicator PR301	FM1	Lee	GL
1	7.4	18	4	8	10	.5	5	4	9	9
2	8.5	19	6	7	11	n.d.	5	10	5	5

* Correspond with ESI Preparations 1 and 2, Table II.
† After hydrolysis (2N HCl, 2 hr) and neutralization.

trate dialyzed overnight against running tap water not exceeding 10°C. The aqueous layer was centrifuged clear and lyophilized. In 3 separate preparations the yield of white amorphous material, soluble in water, was 90-100 mg per liter of packed cells. Analytical data and inhibitory titers for several indicators with 2 preparations of ESI are given in Table III. Hexosamine and reducing sugar values are probably maximal, since samples hydrolyzed with 2N HCl for one to 5 hours gave essentially equivalent values. Electrophoresis of the first preparation at pH 8.6 in veronal 0.1 M showed the presence of one sharply defined major component, and 1-2 much smaller components.‖ The major component showed viral hemagglutination inhibition activity comparable with that of the starting material. The absorption spectra obtained with this soluble erythrocyte stroma inhibitor (ESI) in the direct Ehrlich and Bial reactions are included in Figs. 1 and 2. In preparation 1, derived from a donor of blood group A_1, no blood group A antigen was detectable by inhibition of A-anti-A hemagglutination.

Liberation of chromogen from ESI by concentrated active virus. Two preparations of ESI were treated with concentrated infective influenza virus, one with strain PR301, the other with strain FM1, in the manner described in connection with the P 4 fractions. As shown in Table II, PR301 destroyed the inhibitory capacity for all indicators except FM1. This corresponds with the findings with P4 (Preparations 1, 2, and 3) in which the receptors for FM1 regularly survived treatment with PR301. Concentrated FM1, however, inactivated the inhibitor for all indicators tested. In addition, 27% and 56% of the chromogen was released in dialyzable form from these two preparations by the action of PR301 and FM1, respectively.

Chromatographic analysis. Limited chromatographic analysis of the dialyzable erythrocyte chromogen in several experiments was carried out, using two solvent systems and trichloroacetic acid-orcinol spray as the developing reagent(16). In the first system [iso-butyric acid-water-ammonia(17)] the Rf of ovine sialic acid¶ was .24-.27, and that of the dialyzable chromogen split from both the P4 fraction and the soluble erythrocyte stroma inhibitor .21-.25. A second unidenti-

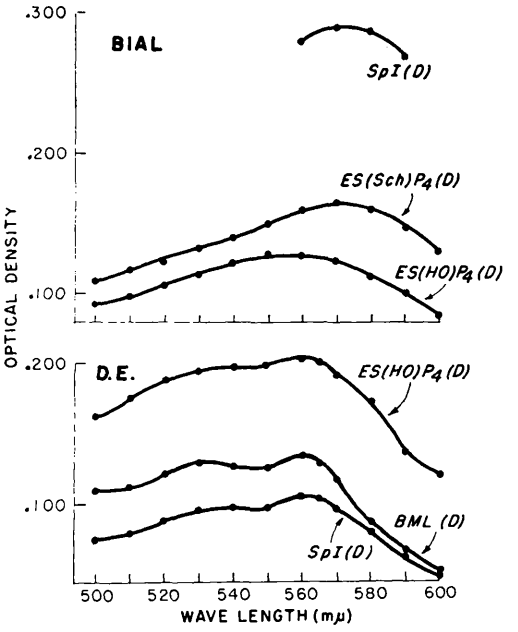

FIG. 3. Absorption spectra of dialyzable chromogen split from erythrocyte stroma concentrate (ES P4), sputum inhibitor (SpI), and human brain mucolipid (BML) by concentrated influenza virus, strain PR301.

‖ Electrophoretic analysis performed by Dr. D. H. Moore.

¶ Kindly supplied by Dr. A. Rosenberg.

fied component with an Rf value of .15-.16 was detected in 2 preparations. In the second solvent system [secondary butanol-acetic acid-acetone-water(18)], the Rf of ovine sialic acid was .44-.46, and that of the dialyzable chromogen .40-.41. The exact significance of these findings is not clear. They suggest, however, that the chromogenic substance split from stroma concentrate as well as from the purified ESI by viral action is some form of free sialic acid.

Discussion. The chromogen found to be liberated in dialyzable form from both crude and purified erythrocyte substrates by action of concentrated infective influenza virus is thought to be sialic acid. By analogy to similar enzymic action of influenza virus on certain soluble mucoids all of which are known to contain sialic acid, it is reasonable to infer that the same chromogen in stroma may be an integral part of the receptor site for the attachment of influenza virus to the intact cell surface. Klenk and Lempfrid(19) have recently reported isolation and crystallization of N-acetyl neuraminic acid [now among the substituted derivatives of neuraminic acid to be referred to as sialic acid(20)] from human erythrocyte stroma and its liberation from this substrate by purified receptor-destroying enzyme (RDE) of *Vibrio cholerae*. These authors have therefore concluded that the erythrocyte receptors for viral hemagglutination consist of sialic acid itself, assuming RDE to have enzymic specificities identical to those of influenza virus. The present studies, in accord with the findings of Klenk and Lempfrid, demonstrate clearly that enzymes of the infective influenza virus particle do indeed act on soluble human erythrocyte components in a similar manner, but the possibility that other complexes, including peptides with sialic acid linkage, might more reasonably serve as sites for attachment and action of *V. cholerae* and influenza virus enzymes has not been excluded. Such possibilities would still be consistent with the liberation of dialyzable sialic acid which, it should be noted, does not by itself inhibit viral hemagglutination. The question of the role of peptides in viral enzyme substrates is being investigated in this laboratory with the use of aminopeptidases of *Clostridium tertium,* culture filtrates of which also happen to show marked receptor-destroying activity(21).

Separation of soluble inhibitors of viral hemagglutination from human erythrocytes has been reported by other workers. DeBurgh, *et al.*(22), using lipid extraction procedures, isolated a fraction in small yield containing 2.6% nitrogen and 48% reducing substance. These authors gave evidence that, under certain conditions, the inhibitory substance was soluble both in water and in organic solvents. The present study also suggests that the purified receptor substance (ESI) includes or is actually a mucolipid, similar to the brain mucolipids described by Rosenberg, *et al.*(12). McCrea(23) isolated an inhibitor from human erythrocytes which gave hexosamine, reducing sugar, nitrogen and phosphorus values not dissimilar to those of the ESI described earlier. Both of the aforementioned investigations, however, were reported before the importance of sialic acid in relation to influenza virus enzymes was generally recognized.

It is of interest in the present studies that some suggestion of a receptor gradient was apparent, in that PR301 failed to inactivate receptors for FM1 either in crude stroma concentrate (P4) or in purified ESI, whereas FM1 inactivated receptors for both strains. Further studies will be necessary to test the validity of this interpretation, and to explore possible quantitative relationships between enzymic activity and receptor destruction in different strains of influenza virus. The greater sialidase(20) activity of RDE on erythrocyte stroma, as reported by Klenk and Lempfrid(19), compared with enzymes of influenza virus acting on similar substrates, as demonstrated in the present study, may be due to the lower total potency of the latter enzyme as used, or to the fact that RDE is soluble whereas influenza enzyme is in particulate form. It is tempting to speculate, however, that strains of virus may differ in the extent to which their enzymic components gain access to sensitive linkages in the receptor sites, whether because of spatial configuration of the substrate or peculiarities of the viral particle, or both.

Summary. The presence in human erythrocyte stroma of a chromogenic substance giving absorption spectra (direct Ehrlich and Bial reactions) identical with those of crystalline sialic acid has been confirmed. A potent water-soluble inhibitor of viral hemagglutination, containing 7-11% by weight of this chromogen, has been isolated by partition dialysis from concentrated human erythrocyte stroma. 30-40% of the chromogen in the crude stroma concentrate and up to 56% of that in the purified inhibitor was rendered dialyzable following treatment with concentrated infective influenza virus, which in most instances also destroyed receptors for indicator viruses. Limited chromatographic analysis indicated that the dialyzable chromogen is most probably sialic acid. These observations represent the first direct demonstration of split products resulting from enzymic action of influenza virus on human erythrocyte components.

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Growth of Human Tumors in Hibernating Hamsters.* (23402)

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Previous studies by Lyman and Fawcett (1) have shown that the growth rate of homologous tumor transplants in the hamster cheek pouch was diminished when the animals were induced to hibernate. The effect of the low temperature of hibernation (5°C) on the tissue of an animal which does not hibernate has not been previously tested. Lyman and Fawcett suggested that such a tissue might be less resistant to cold since there are apparently innate differences between the cellular physiology of hibernating and non-hibernating mammals. With the recent development of technics for transplanting human tumors

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