on water; adrenalectomized DCA-implanted rats on 0.9% sodium chloride solution. These rates correspond to those predicted on the basis of the known effects of the secretion of the adrenal cortex and the concentration of extracellular sodium on brain excitability. Cortisone and adrenocortical extract (ACE) lowered the elevated EST of adrenalectomized DCA-implanted rats which were given a solution of sodium chloride to drink by 8.5% in 6 days. Following withdrawal of cortisone or ACE the EST returned to pretreatment levels in 11 days. Cortisone lowered the elevated EST of adrenalectomized DCA-implanted rats given water to drink by 10% in 3 days; following withdrawal of cortisone the EST returned to the pretreatment level in 2 days. It has been suggested that the results obtained provide evidence for the concept that cortisone and DCA compete for strategic loci in target cells.

Received December 5, 1950. P.S.E.B.M., 1951, v76.

Hemagglutination by Col-MM-Virus. (18390)

SVEN GARD AND LEO HELLER. (Introduced by Albert B. Sabin.)

From the Department of Virus Research, Caroline Institute, Stockholm, Sweden.

Members of the EMC group of viruses(1,2) are capable of agglutinating sheep erythrocytes as first shown by Hallauer(3) and confirmed by Bremer and Mutsaars(4,5), Verlinde and de Baan(6) and Olitsky and Yager (7). Hemagglutinating capacity of the GD VII strain of Theiler's virus against human group O red blood cells was reported by Lahelle and Horsfall(8) and of Japanese B encephalitis virus for both sheep and chick erythrocytes by Sabin and Buescher(9). In all those cases, the hemagglutination could be inhibited by homologous immune sera.

Working with the Col-MM strain(10) of EMC virus, we have tried to establish a standard technic for assay of hemagglutination-inhibiting antibody for the purpose of studying the importance of this group of viruses as cause of disease in man(11-14). The difficulties encountered in virus hemagglutination (HA) experiments in general can be referred to the following complications: (a) the insensitivity of hemagglutination as a means of demonstrating the presence of virus, the hemagglutinating activity being inferior to infectivity by several powers of ten, (b) the appearance of false positives, caused by crude tissue extracts(7), bacterial contamination, so called spontaneous agglutination, etc. In order to increase the sensitivity of the HA reaction we have used very dilute cell suspensions [*cf.* Whitman(15)], the erythrocyte concentration ultimately adopted being 0.05%. Under such conditions the effects of salt agglutination and similar disturbances are more pronounced and the system has to be stabilized. This can be achieved by addi-

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tion of serum, albumin, or other proteins. We have found extracts of normal mouse or sheep brain, clarified by medium speed centrifugation, to be excellent stabilizers, superior to serum and bovine plasma albumin in giving more clear-cut negative patterns in the absence of virus and, thus, sharper endpoints, especially in hemagglutination-inhibition titrations.

In the experiments to be reported infected mouse brains served as source of virus and a 10% sheep brain extract, clarified by centrifugation for 30 minutes at 12000 G, was used as diluent. The red cells were stored in Alsever's or ACD I solution, washed and resuspended in veronal-buffered saline of pH 7.3-7.4. Salk's method of recording agglutination was used throughout.

Hemagglutination-inhibition (HI) by electrolytes. Inhibitors of the type described by Sabin and Buescher(9), effective against Col-MM hemagglutinin, were not found in extracts of normal or infected brain. Neither was hemagglutination inhibited by a number of pseudomucinous ovarian cyst fluids* of blood groups A and O, showing a high inhibitor activity against influenza virus hemagglutinin. Unlike the case of Japanese B virus(9) Col-MM virus hemagglutination proved to be independent of pH in the range of 6.0-9.0. On the other hand the ionic composition of the medium was of the utmost importance. Hypertonic salt solutions tended to suppress hemagglutination. As to inhibiting capacity, however, there was a pronounced difference between positive and negative ions. Whereas mono- and divalent cations showed no obvious effects, multivalent anions caused a considerable inhibition, roughly proportionate to their charge. The results recorded in Fig. 1 show, for instance, that hexametaphosphate in a concentration of 0.0002M inhibited completely 4 HA units of virus.

According to Whitman(15) the amount of specific immune serum needed for inhibition of influenza A virus is proportionate to the dose of virus, indicating the combination of virus and antibody as a principal factor in

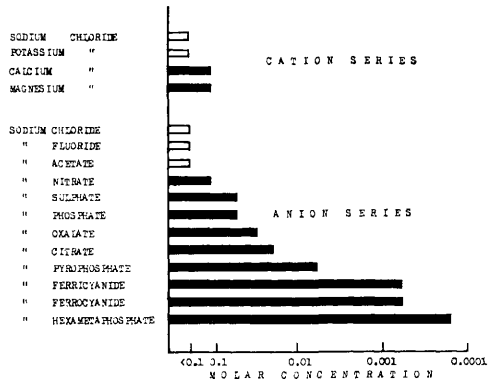

FIG. 1.

Inhibiting effect of different ions on Col-MM virus hemagglutination.

the mechanism of inhibition. In the experiment, summarized in Table I, inhibition by hexametaphosphate and immune serum was compared from this point of view. The HI titers of the 2 reagents were determined against 6 different virus doses and 4 different red cell suspensions. As shown in Table I there was an almost perfect rectilinear relationship between virus and inhibiting serum dose. Except for certain irregularities in systems with very dilute cell suspensions and large doses of virus, an increase in the concentration of virus used in the test caused an exactly corresponding decrease in HI-titer of the serum. On the other hand, the HI-titer of hexametaphosphate was largely independent of the amount of virus present. Thus a 32-fold decrease in virus concentration led to an 8-fold increase in HI-titer in a 0.2% cell-suspension and only 2-fold in 0.025% suspensions. The results indicate that hexametaphosphate acts upon the red cell rather than upon the virus. This interpretation received additional support in the results of virus neutralization experiments in which maximal non-toxic concentrations of Na-hexametaphosphate (0.1 ml of a 0.05-molar solution), substituted for serum in an otherwise classical intraperitoneal mouse neutralization test(16,17) failed to neutralize 10 and 50 LD_{50} respectively of Col-MM virus-amounts,

* Obtained through the courtesy of Dr. R. Grubb, Bacteriol. Institute, University of Lund, Sweden.

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TABLE I. Hemagglutination-inhibition Titers of Sodium Hexametaphosphate and of Rabbit Col-MM antiserum Titrated Against Varying Amounts of Col-MM Virus Hemagglutinin and of Sheep Red Cells.

Final conc. of red cells, %	Final dilutions of virus:										Increase in HI-titer corresponding to 32-fold decrease in virus:	
	1:40	1:80	1:160	1:320	1:640	1:1280	1:40	1:80	1:160	1:320	1:640	1:1280	Na-HMP*	Serum
	Sodium hexametaphosphate (final molarity)					Hemagglutination-inhibition titers of:	Col-MM antiserum (final dilution)							
.2	M/800	M/800	M/800	M/1600	M/3200	M/6400	1:64	1:128	1:256	1:512	1:1024	1:2048	8-fold	32-fold
.1	M/800	M/800	M/800	M/1600	M/1600	M/3200	1:64	1:128	1:256	1:512	1:1024	1:2048	4-fold	32-fold
.05	M/800	M/800	M/800	M/1600	M/1600	M/1600	1:64	1:128	1:256	1:512	1:1024	1:2048	2-fold	32-fold
.025	M/800	M/800	M/800	M/1600	M/1600	M/1600	1:32	1:64	1:256	1:512	1:1024	1:2048	2-fold	64-fold

* Na-HMP = Sodium hexametaphosphate.

by several powers of 10 smaller than those inhibited *in vitro* by c:a 100 times lower concentrations of the same salt.

The following experiment illustrated further the differences between the two types of inhibitors. In one set of tubes serial dilutions of the inhibitor were mixed with a fixed amount of virus. The mixtures were kept at +4°C for one hour, after which the red cell suspension was added. In another set the order of mixing was reversed, the virus being allowed to act upon the red cells for one hour before addition of the inhibitor. The results are presented in Table II. The table shows that the inhibiting effect of immune serum was considerably reduced once the combination between virus and cell had taken place. As a certain activity remained even under these conditions, either immune serum has the capacity to a certain extent to split the virus-cell combination, or else a partial dissociation of the latter is taking place in connection with the slight rise in temperature during manipulation. On the other hand, the effect of hexametaphosphate was exactly the same, irrespective of the order of mixing.

Following the same line of approach we tested also a crude preparation of thymus tetranucleotide [a product of controlled and specific enzymic hydrolysis of thymus desoxypentosenucleic acid(18)], chosen as being a more 'physiologic' polyvalent negative ion, and found it to be an exceedingly strong inhibitor, active down to the concentration of about 0.1 mg/ml. This corresponds to c:a 0.0001-molarity, assuming an average tetranucleotide size of the molecules of this no doubt heterogeneous oligonucleotide preparation. At the same time muscle adenylic acid (AMP)—a representative of the class of mononucleotides which, due to the very low second ionization constant of their phosphoric acid rest, must be assumed to appear in the neighborhood of pH 7 as prevalently monobasic—was found to be devoid of inhibiting activity at concentrations as high as 15 mg/ml (the highest tested), corresponding to c:a 0.05-molarity. Two crude preparations of

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TABLE II. Reversibility of the Col-MM Virus Hemagglutination by Homologous Immune Antisera and by Sodium Hexametaphosphate (Na-HMP).

Inhibitor	No. HA-units of virus	Initially mixed:	After 1 hr at +4°C added:	HI-titer	Difference in titer
Col-MM	4	Virus + antiserum	RBC	1:1920	
Antiserum	4	" + RBC	Antiserum	1:320	6-fold
Na-Hexameta-	4	" + Na-HMP	RBC	1/3200-M	
phosphate (Na-HMP)	4	" + RBC	Na-HMP	1/3200-M	None

Volumes used: Col-MM virus, 0.2 ml; antiserum resp. Na-HMP-dilutions, 0.4 ml; 0.3% sheep red blood cells (RBC), 0.2 ml.

ribose and desoxypentose nucleic acids (the former a commercial yeast nucleic acid 'Boehringer' and the latter a low viscosity product prepared from calf spleen) both inhibited, the latter, however, about ten times more than the former (on an absolute concentration basis). These experiments must be repeated with better defined materials before any definite conclusions can be drawn.

Serum hemagglutination-inhibition. For elaboration of a standard HI-technic the following observations were important. In a study on the stability of virus and antiserum we found that the hemagglutinating titers of Col-MM virus suspensions (10% suspensions of infected mouse brain in 0.85% saline, clarified by 30 minutes spinning at 12,000 G), stored at –60°C, used to show a slight but rather consistent increase during the first few days. On further storage, however, the hemagglutinating capacity decreased continually to disappear completely after a period of several months. A parallel loss of infectivity was also demonstrable. Generally, virus suspensions older than 4-6 weeks could not be used for the HI-tests. Inactivation proceeded more rapidly on storage at higher temperatures as well as upon repeated thawing and freezing. A lyophilized rabbit hyperimmune serum showed a high and constant HI-titer when tested against freshly harvested virus hemagglutinin but gradually lower titers in tests with aging virus preparations. A plausible explanation of this phenomenon is the following. A loss of hemagglutinating activity and infectivity is not necessarily accompanied by a corresponding decrease in the capacity of combining with specific antibodies. One hemagglutinating unit of a partially inactivated virus comprises a larger quantity of virus protein than the same unit of a 100% active virus, and might therefore have a much larger antibody-combining power. Consequently, the HI-titer of a given serum is not a fixed quantity but dependent upon the amount and the state of the virus used in titration. Two different titrations can be compared only if they are performed simultaneously with the same virus preparation and dose. We have found it necessary, therefore, when examining unknown sera, to run simultaneously a titer on a known standard serum and refer the inhibiting activity of a serum to that of the standard rather than to give the individual titer values. The technic finally adopted was as follows.

Methods. Sheep red cells (RBC) were stored and treated as described above. The use of phosphate buffer was omitted on account of its inhibitory effect and veronal-saline substituted for it. A 10% sheep brain extract clarified by centrifugation at 12,000 G was used as diluent for the virus hemagglutinin. A standard dose of 4 HA units was chosen. Sera to be examined were inactivated for 30 minutes at 56°C and then absorbed in the cold with washed packed sheep red cells. Occasionally the absorption had to be repeated for removal of all hemagglutinins. The standard serum, a rabbit hyperimmune serum, was likewise inactivated at 56°C and absorbed with sheep red cells and normal mouse brain. It was lyophilized in exactly measured amounts of 0.2 ml, sealed off in a nitrogen atmosphere and stored at –15°C. Tests were set up with 0.2 ml of serial dilutions of serum, 0.1 ml of virus dilution, containing 4 HA units, and, after ½ hour at room

TABLE III. Results of HI-Titrations on Human Sera.

Group of patients	No. of sera tested	HI-index:	No. and (%) of sera within each group classified as: Negative <1	Weak 1-4	Positive 8	16	32	>32
1. Neurotropic infections (paralytic and non-paralytic poliomyelitis, aseptic meningitis, encephalitis, etc.)	384		281 (73.2%)	55 (14.3%)	29	10	7	2
					—(48 (12.5%)—			
2. Various diseases (no involvement of the central nervous system)	39		39 (100.0%)	—	—	—	—	—
3. Normal controls	146		129 (88.4%)	16 (10.9%)	1 (0.7%)	—	—	—

temperature, 0.1 ml of a 0.2% RBC suspension was added. The racks were placed in the refrigerator and readings according to Salk were made after 4 hours and again the next day. The following controls were included: (a) test serum + RBC (absence of serum hemagglutinins) (b) HA-titration of the virus used, and (c) HI-titer of the standard serum.

The result was expressed as inhibiting activity in per cent of that of the standard serum = *HI index*. Sera with indices below 1 were classified as negative, reactions in the range of 1 to 4 were considered as doubtful or weak, and values of 8 or above were recorded as positive.

Results. The material so far examined consisted of 384 sera from patients showing involvement of the central nervous system (paralytic and non-paralytic poliomyelitis, aseptic meningitis, encephalitis, etc.), 39 sera from patients with various diseases showing no such involvement, and a control group of 146 sera collected from blood donors and healthy pregnant women in a prenatal clinic.

The results can be summarized as follows (Table III). In the first group 73.2% were negative, 14.3% showed weak and 12.5% definitely positive reactions. In the second group only negative reactions were observed. In the third group, finally, there were 88.4% negative, 10.9% weak reactions and one out of 146 sera (0.7%) gave an index of 8.

The difference between the first group on one hand and the remaining groups on the other is highly significant, the probability of its being due to chance being less than 0.001.

A number of human sera with high HI-indices were tested for virus neutralizing capacity. In parallel tests rabbit hyperimmune serum diluted to give corresponding HI-indices was employed. Neutralization was performed intraperitoneally in mice against 15-50 MLD of virus. The rabbit serum gave complete neutralization, *whereas all human sera failed to neutralize.*

Discussion. Olitsky and Yager (7) reported that combination of virus and red cells occurred only in the cold and that complete dissociation of this combination took place when the temperature was raised to about + 20°C, without apparent changes in the properties of either virus or cells. This observation, which we can largely confirm, seems to indicate that mainly low energy bonds are involved in the reaction. The fact that polyvalent anions have a strong inhibiting effect points in the same direction. It seems probable that the ions do not act upon the virus itself but on the cell, in some way influencing the reactivity of the receptor groups.

The preliminary results of our experiments with organic compounds of the nucleotide-nucleic acid group could be interpreted as effects of organic anions, in accordance with the previous findings. The quantitative difference between thymus and yeast nucleic acid might be indicative of some additional, more specific factor but has to be confirmed with more well-defined products before any conclusions could be drawn.

Whatever the mechanisms of these phenomena may be, it is evident that the Col-MM virus hemagglutination is extremely sensitive to changes in the composition of the medium.

Therefore, appearance of serum inhibitors cannot be considered by itself as evidence of infection with a member of the EMC group of viruses. As a matter of fact, the failure of human sera to neutralize the virus seems to prove that the inhibiting capacity is not due to the presence of specific antibodies and, thus, to exclude definitely the assumption of EMC virus as an etiologic factor in the cases in question. The inhibitor found might still be an antibody, although heterologous, or it might be a non-specific substance appearing in the blood in connection with disturbances of the central nervous system. This question will be the subject of future experiments.

Summary. 1. Hemagglutination of sheep red blood cells by the Col-MM strain of the EMC virus was inhibited by polyvalent anions, the inhibitor titers (HI-titers) of the different negative ions being roughly proportionate to their net electrical charge. 2. Whereas the HI-titer of specific antibody was inversely proportionate to the amounts of virus, indicating a stoichiometric combination of virus and antibody, the HI-titers of sodium hexametaphosphate (6-valent anions) were only slightly influenced by the quantity of virus added. The order of mixing of the reactants was of great importance for the serum inhibition but without effect on the activity of hexametaphosphate. 3. The strong hemagglutination-inhibiting effect of sodium hexametaphosphate was not paralleled by any virus neutralizing activity. 4. A standard serological HI-test, based on the results of a study of the Col-MM virus hemagglutination, is described. Applied on 569 human sera, this method gave a high incidence (12.5%) of strongly positive hemagglutination-inhibition titers among 384 sera obtained from patients showing distinct involvement of the central nervous system. Weakly positive reactions were encountered in 14.3%. A control group, consisting of 146 sera collected from healthy humans showed only 0.7% strong and 11% weak reactions, while 39 sera from patients with various ailments but apparently free from neurotropic infections were negative throughout. 5. Even strongly inhibiting human sera failed to neutralize Col-MM virus in animal experiments.

Received December 9, 1950. P.S.E.B.M., 1951, v76.

Effect of Various Levels of Vitamin B_{12} Upon Growth Response Produced by Aureomycin in Chicks. (18391)

E. L. R. STOKSTAD AND T. H. JUKES.

From the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

It was reported by Stokstad and coworkers(1) that the growth of vit. B_{12}-deficient chicks was increased by the addition of a crude supplement prepared from fermentation with *Streptomyces aureofaciens*. The presence of vit. B_{12} and B_{12b} in this fermentation was subsequently demonstrated (2) and it was shown that the supplement also contained aureomycin which was found to increase the growth rate of chicks on diets either adequate(3,4) or deficient(5) in vitamin B_{12}. It thus became evident that either aureomycin or vit. B_{12} would produce a growth response in chicks on an all-vegetable diet and some evidence was obtained(5) which suggested that aureomycin had a spar-

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