17309. Hemagglutination by Columbia SK, Columbia MM, Mengo Encephalomyelitis and Encephalomyocarditis Viruses: Experiments with Other Viruses.

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During the course of a study on hemagglutination by certain neurotropic viruses, a study suggested by the findings reported in the foregoing paper,[1] the writers' attention was directed to two reports. One[2] stated that the Lansing strain of poliomyelitis virus, and the other[3] that the Columbia SK (Col SK) and Columbia MM (Col MM) viruses agglutinated sheep erythrocytes, the agglutination being inhibited by specific antisera.

In our own work no hemagglutination by the Lansing strain was found; Hallauer[3] also reported failure. On the other hand, hemagglutination of sheep RBC by Col SK and Col MM viruses[3] was not only confirmed in the present investigation but a similar specific reaction was also obtained with Mengo encephalomyelitis (ME) and encephalomyocarditis (EMC) viruses.

This paper reports the results of these tests as well as attempts to disclose agglutination of sheep red cells and several additional types of erythrocytes by still other neurotropic viruses. Furthermore, there will be described an agglutinin for erythrocytes deriving from several species of animals present in suspensions of normal mouse brain, as well as an inhibitor of agglutination contained in the tissue suspensions and also in normal serum.

Hemagglutination of Sheep Erythrocytes by Col SK, Col MM, ME, and EMC Viruses. Dick and Taylor[4] have employed solutions of crystalline bovine plasma albumin (BPA) as a medium for preservation and for dilution of several viruses, among which were influenza, yellow fever, Lansing and ME. A 0.1 or 0.2% solution of the crystals in buffered saline solution, filtered through a Seitz apparatus generally sufficed for ordinary laboratory purposes, especially for dilution in titration tests.[4] It was also found in this laboratory that for the several viruses employed in the present study BPA was a satisfactory vehicle and the advantages of utilizing a clear, nonagglutinating solution, which also preserved the titer of a virus in tests for hemagglutination, were apparent. Consequently virus-infected mouse brains, (20%) were suspended preferably in 0.1% BPA although saline solution or 10% rabbit serum could also be employed. Such suspensions were used in the fresh state; or if stored, were kept frozen in a mechanical, electrically operated freezer at -20 to -25°C and thawed just before use. Dilutions of virus for hemagglutination were made, however, with buffered saline solution, 0.85% NaCl, 0.05 M phosphate and pH 7.6.

The procedure of the test was as follows: The fresh or thawed viral suspension consisting of 20% brain tissue was centrifuged for clarification at 2,000 rpm for 5 minutes. 0.4 ml of the supernate was added to the first 2 of a series of 10 to 15 tubes. Buffered saline solution in equal amount was introduced into all tubes except the first to make a 2-fold dilution in a final volume of 0.4 ml in the second and successive tubes. To each virus dilution was added 0.4 ml of 0.5% washed sheep RBC suspended in buffered saline solution, thus securing final dilutions of virus of 1:10 to 1:5, 120, or higher, and of the erythrocytes in each tube, 0.25%. Cells were prepared from fresh bleedings and stored in modified Alsever's fluid (ACD);[4a] as such they could be kept for about 1 month in the ice

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[1] Lahelle, O., and Horsfall, F. L., Jr., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 713.

[2] Bremer, A., and Mutsaars, W., *C. r. Soc. biol.*, 1948, **142**, 1194.

[3] Hallauer, C., 4th Internat. Cong. Microbiol., July 20-26, 1947, Copenhagen, 1949, p. 257.

[4] Dick, G. W. A., and Taylor, R. M., *J. Immunol.*, 1949, **62**, 311.

[4a] Rapoport, S., *J. Clin. Invest.*, 1947, **26**, 591.

TABLE I.
Hemagglutination of Sheep Cells by Columbia SK, Columbia MM, Meningo Encephalomyelitis and Encephalomyocarditis Viruses, Held for 120 Min. at 5°C.

	Virus or control materials	Reciprocal of final dilution of virus or of normal mouse brain 10	20	40	80	160	320	640 to 5,120	RBC of types other than sheep, plus virus (10 to 5,120 dils.)
Test	Col SK	4	4	2	±	0	0	0	0
	Col MM	4	4	2	0	0	0	0	0
	ME	4	3	3	3	2	±	0	0
	EMC	4	4	4	3	2	1	0	0
	West equine	0	0	0	0	0	0	0	0
	East "	0	0	0	0	0	0	0	0
	GDVII	0	0	0	0	0	0	0	+ with human-o cells only
Controls	Normal mouse brain	0	0	0	0	0	0	0	See text
		sheep cells							Cells alone
	Saline soln.	0							0
	BPA	0							0

box; after washing, however, not longer than 5 days. Hamster erythrocytes were prepared from fresh bleedings and were not satisfactorily stored; they were proved useless if kept in ACD for periods longer than 4 days. The tubes were shaken, kept for 60-120 minutes at 5°C and then read. It should be emphasized here that false positives, *i.e.*, nonspecific reactions, easily obscured the results especially since mouse-brain suspensions were employed; the cause of this difficulty will soon be given. Hence tests should include a) a control on the virus suspension, namely, normal mouse brain suspension and b) antiserum to determine the specificity of hemagglutination. Generally the methods here described and the scale of reading agglutination, except for certain modifications, follow those already described.[1,5] It should be stressed here also that even slight variations in technic sometimes brought about irregular results —a fact which applies also to the standard test.[5]

Table I shows the outcome of one of several similar experiments. The selective agglutination of sheep RBC by Col SK, Col MM, ME and EMC viruses is noted. It is not surprising to find such uniformity of reaction exhibited by the 4 viruses since it has already been found by Warren and Smadel[6] and by Dick[7] that there is a close relationship among the members of this group as proved by the results of serological, immunological and other biological studies.

With these 4 viruses the hemagglutination was carried out best at 5°C for the reason that spontaneous elution or dispersion of virus from the erythrocytes occurred at room (23°C) or incubator (37°C) temperatures so that at the higher temperature little or no agglutination was visible. This reversibility of the agglutination by means of increasing the temperature could be carried out with the same materials for an indefinite number of times, or as long as sufficient virus survived the process to show its hemagglutinative capacity. In this respect, the present group of viruses behaved as did the GDVII virus.[1]

Specificity of Hemagglutination. The next investigation related to the specificity of the hemagglutination for sheep erythrocytes by Col SK, Col MM, ME, and EMC viruses. The procedure followed the established principles[5] of a preliminary titration to determine the hemagglutination titer of the virus to be tested and of selection of a dilution of it which represented not less than 4 and not more than 8 agglutination units. 0.2 ml of this dilution of virus was added to 0.2 ml of antisera, then 0.4 cc of the cells—no preliminary

[5] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49; Smadel, J. E., in Viral and Rickettsial Infections of Man, ed. T. M. Rivers, J. B. Lippincott Co., Philadelphia, 1948, Chap. 3, pp. 77-82.

[6] Warren, J., and Smadel, J. E., *J. Immunol.*, in press.

[7] Dick, G. W. A., *J. Immunol.*, in press.

TABLE II.
Hemagglutination-Inhibition by Antisera: Col SK, Col MM, ME and EMC Viruses and Sheep Cells.

Virus	Antiserum*	Virus. agg. units	Reciprocal of final dilution of serum							
			40	80	160	320	640	1,280	2,560	5,120
Col SK	Col SK	4	0	0	0	0	0	0	0	4
Col SK	NRS†	4	4	4	4	4	4	4	4	4
Col MM	Col MM	4	0	0	0	0	0	1	2	3‡
Col MM	NRS	4	4	4	4	4	4	4	4	4
ME	ME	8	1	0	0	0	0	0	3	4
ME	NRS	8	4	4	4	4	4	4	4	4
EMC	EMC	8	1	0	0	0	0	2	4	4
EMC	NRS	8	4	4	4	4	4	4	4	4
ME	WEE§	8	4	4	4	4	4	4	4	4
EMC	WEE	8	4	4	4	4	4	4	4	4

* All antisera were prepared by injecting rabbits repeatedly with mouse-brain virus.
† NRS = normal rabbit serum.
‡ The titration continued as follows: 10,240 read 3; 20,480 read 4.
§ Western equine encephalitis antiserum.

period of incubation of antisera and virus was found necessary. The antisera used were prepared by injecting rabbits subcutaneously 3 times at weekly intervals with 1, 2, and 3 respectively, fresh or frozen virus-infected mouse brains. All serum whether immune or normal was inactivated by heating at 56°C for 30 minutes. The virus was kept constant at 4 or 8 units per tube and the serum was diluted 2-fold beginning with 1:10, thus the final dilutions of serum became 1:40 to 1:20,480, since 10 dilutions were usually tested. The test was read after 60 minutes at 5°C. The reading of the hemagglutination inhibition followed standard methods.[5]

Table II demonstrates one of the tests. It will be observed that not only did the antisera inhibit specifically the hemagglutination of sheep cells by the Col SK group of viruses but there was evidence of clear-cut cross-reactions among members of this group of viruses. Table III is presented to demonstrate the results of a test on inhibition of hemagglutination, the titer of antisera and certain cross-reactions among the members of the Col SK group of viruses.

TABLE III.
Tests Showing Hemagglutination-Inhibition Titers of Antisera Against Col SK Group of Viruses and Certain of the Cross-Reactions.

Virus	Antiserum	Agglutination-Inhibition titer
Col SK	Col SK	1:2,560*
	Col MM	1:640
	ME	1:1,280
	EMC	1:1,280
ME	ME	1:2,560
	EMC	1:320
EMC	EMC	1:640
	Col SK	1:640

* The dilutions represent the highest dilution of antiserum preventing agglutination (Table II).

It is concluded therefore that the Col SK, Col MM, ME and EMC viruses agglutinate sheep RBC specifically; they exhibit cross-agglutination inhibition among the individuals of the group and since other neurotropic viruses do not agglutinate sheep cells, as will be shown immediately, these 4 infective agents can be looked upon as having a generic relationship and a common antigenicity. Thus support is given to the findings of Warren and Smadel[6] and of Dick[7] who produced solid evidence from a wholly different approach to the problem of interrelationship of the 4 agents.

Since the hemagglutination is characteristic, it can apparently be utilized for the identification of the viruses of the Col SK group and for measurement of the antibody content of antisera against any of the 4 agents. Finally, since all of the individual members of the group agglutinate sheep RBC in the cold, elute or disperse spontaneously and rapidly at moderate elevations of temperature, and when

freed from the erythrocytes are as active as they were originally, hemagglutination may be useful for purposes of adsorption, without inactivation, of the active agents, just as can be done with the GDVII virus.[1]

Agglutination Tests with Other Neurotropic Viruses and a Variety of RBC. A wide variety of erythrocytes other than those of sheep, namely, human O, chick, horse, hamster, dog, cat and guinea pig, were tested for agglutination by the 4 viruses of the Col SK group; the tests failed. In addition, the various erythrocytes just mentioned, including sheep cells, were tested for agglutinability by numerous neurotropic viruses. The viruses employed were:

Eastern equine encephalitis	Theiler (FA strain)
Western equine encephalitis	Theiler (TO strain)
Venezuelan equine encephalitis	poliomyelitis (Lansing strain)
Japanese B encephalitis	poliomyelitis
St. Louis encephalitis	(MEFl strain)
Russian Far East encephalitis	West Nile
vesicular stomatitis (New Jersey strain)	rabies
vesicular stomatitis (Indiana strain)	lymphocytic choriomeningitis
	herpes simplex
	louping ill

The results of over 100 experiments can be summarized by stating that no specific clumping of any of the types of erythrocytes by any one of the viruses mentioned was detectable. Now and again agglutination was seen but further study revealed it to be nonspecific, most often owing to the physical or particulate condition of the mouse-brain suspension. It was shown that a) normal mouse-brain suspensions produced similar hemagglutination; b) centrifugation to a degree which clarified the suspension but did not sediment the virus from the supernate served to abolish the agglutinative capacity of the supernate, c) antisera failed to inhibit the reaction, d) the reaction was not reversible, e) the aggregations formed did not resemble the "soft", clinging, fine agglomerations as was seen in the hemagglutination by Col SK group of viruses just described. On the contrary, the aggregates were usually "hard", coarse, irregularly sized and shaped, and sometimes were surrounded by a narrow zone of slight hemolysis.

In view of the fact that GDVII virus exhibits agglutination only of human-O RBC[1] and the Col SK group of viruses only of sheep cells, one may well question the meaning of the negative results obtained with the other neurotropic viruses and the kinds of erythrocytes used in the present investigation. Since agglutination is so selective with respect to cells used, it will not be surprising to find one or another of these viruses yielding positive results with erythrocytes deriving from species not as yet tested.

Agglutinative Capacity of Normal Mouse Brain Suspensions for Dog, Cat and Guinea Pig RBC. During the course of the present study, it was noted that suspensions of normal mouse brain agglutinated to a low degree, dog, cat, and guinea pig RBC. The hemagglutination titers were generally 1:10, less often 1:20, rarely 1:40, and of 15 samples in one instance only, 1:320. This reaction was observed after 60 minutes at 5°C, but the maximum titer was reached, however, at room temperature. The reaction was not reversible: there was no visible phenomenon similar to that of spontaneous elution. Moreover, antisera prepared by immunizing rabbits against normal mouse brain had no inhibitory effect on the agglutinative power of the normal mouse brain. The agglutination thus produced revealed, therefore, characteristics unlike those of the viruses of the Col SK group. It is clear, however, that since neurotropic viruses are often employed in the form of infected mouse brain, the occurrence of this nonspecific hemagglutination should be reckoned with in experimental studies.

Presence of an Agglutinin and an Agglutination-Inhibitor for Hamster Cells in Normal Mouse Brain. It was also disclosed during the course of the present investigation that there exists in normal mouse brain, and therefore in virus suspensions prepared with infected mouse brain, an agglutinin as well as an agglutination-inhibitor (HI) for hamster erythrocytes, both being present in the same suspensions at the same time.

TABLE IV.
Presence of Hemagglutination-Inhibitor and Agglutination of Hamster RBC by Normal Mouse Brain (NMB) and Virus-Infected Mouse Brain (120 min. at 5°C).

Material used	Dilution of brain tissue or of antiserum*											
	40	80	160	320	640	1,280	2,560	5,120	10,240	20,480	40,960	81,920
NMB	0	0	0	0	0	3	4	4	4	4	4	4
NMB + its antiserum	0	0	0	0	0	0	2	2	3	3	—	—
NMB + NRS*	0	0	0	0	±	1	4	4	4	4	—	—
West equine virus	0	0	0	1	3	4	4	4	4	4	4	2
West equine virus + its antiserum	0	0	0	1	2	4	4	4	4	—	—	—
NMB heated, 56°C, 10 min.	4	4	4	4	4	4	4	4	4	4	4	4
NMB heated, 56°C, 30 min.	4	4	4	4	4	4	4	4	4	4	2	0

* NRS = Normal rabbit serum; in an HI test, 8 units of normal or virus-infected mouse brain was used in each tube; reciprocal of final dilution is given.

The agglutinin for hamster cells present in normal and virus-infected mouse brain became evident at ice box or room temperature, maximal after 2 hours' incubation. The agglutinated masses of hamster cells which formed were more minute, delicate and evenly dispersed along the sides of the test tube than were the aggregates formed by the Col SK group of viruses. Specific antisera did not inhibit the hamster-erythrocyte agglutination to any greater extent than did normal serum of the same species. As will be shown later all sera contained a nonspecific inhibitor. It is therefore plain that this agglutinin has characteristics which differ from those shown by the Col SK group of viruses in the presence of sheep cells. The hamster-RBC type agglutination also differs from that observed when normal or infected mouse brain reacted with dog, cat or guinea pig erythrocytes.

The HI exerted its influence in dilutions of 1:40 to 1:160, rarely (3 of 18 titrations) as high as 1:640. After the HI was diluted out hemagglutination was then visible and showed itself in dilutions usually up to 1:80,000 and sometimes higher (Table IV). The HI was active both at ice-box and room temperatures and the maximum titers were noted after 2 hours' incubation. The inhibitor was thermolabile and could be inactivated by heating mouse-brain suspensions at 56°C for 10 to 30 minutes at which temperature the agglutinin was not affected (Table IV). Sodium citrate 2.5% did not neutralize its effect as it does in the case of PVM and other viruses.[8] It could not be sedimented out of a suspension of mouse brain at 3,000 rpm for 10 minutes and rabbit antisera prepared by repeated injection of normal or virus-infected mouse brain failed to reduce its titer of activity.

Nonspecific HI in Certain Normal Serum and Antisera. Antisera prepared in rabbits, guinea pigs, or monkeys, and the respective normal sera of these species possessed the capacity to inhibit hamster-cell agglutination by normal or virus-infected mouse-brain suspensions. Thus the nonspecific inhibition could be demonstrated in dilutions of sera up to 1:1,280 (Table IV). When normal guinea pig or rabbit serum was heated at 65°C for 30 minutes the contained nonspecific inhibitor was not thereby inactivated. It has been previously reported[8,9] that such treatment of serum can inactivate the inherent nonspecific inhibitor of hemagglutination by other viruses.

8 Ginsberg, H. S., personal communication.

9 Ginsberg, H. S., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1949, **89**, 37.

Summary of Nonspecific Agglutinins and Inhibitors. In sharp contrast to the clearly defined, specific agglutination of sheep red cells by the Col SK group of viruses on one side, and the failure of many other neurotropic viruses to show hemagglutination on the other, is the existence of the following nonspecific elements:

a) An agglutinin for dog, cat and guinea pig erythrocytes is contained in normal, or virus-infected mouse brain.

b) An agglutinin for hamster red cells is present in normal or virus-infected mouse brain.

c) A thermolabile inhibitor of agglutination of hamster RBC exists concomitantly with the agglutinin for the hamster cells just mentioned, in normal or virus-infected mouse brain.

d) A thermostable inhibitor is present in normal monkey, rabbit and guinea pig serum, consequently in antisera as well, which prevents agglutination of hamster cells.

The nonspecific reactions just described require further study for the identification of the agglutinins and the inhibitors present in mouse brain suspensions and in normal serum. For the moment, the use of erythrocytes deriving from hamsters, dogs, cats and guinea pigs for agglutination by neurotropic viruses in the form of mouse-brain suspensions would appear to require caution. Since all the standard hemagglutination tests, now routine in laboratory practice, require careful control of the variable employed, similar precautions are needed as well for the Col SK, Col MM, ME, and EMC viruses.

Conclusions. Evidence has been brought forward to indicate that Columbia SK, Columbia MM, Mengo encephalomyelitis and encephalomyocarditis viruses agglutinate sheep red cells. It is therefore possible to identify these viruses by means of hemagglutination and to measure the antibody content of antisera. Since the viruses show characteristic spontaneous elution or dispersion from the erythrocytes, the method can be used for purposes of selective adsorption of the viruses without loss of their hemagglutinative activity. Moreover, the uniformity of the hemagglutination reaction shown by the 4 viruses and the cross-inhibition that exists among them supports the findings of Warren and Smadel[6] and of Dick[7] that these viruses are similar in many repects and are of the same group.

Seventeen other neurotropic viruses were tested for their capacity to agglutinate characteristically erythrocytes deriving from sheep, man (group 0), chicken, horse, hamster, dog, cat and guinea pig; these tests failed.

Another phenomenon that was observed is the nonspecific agglutination of dog, cat and guinea pig erythrocytes by normal or virus-infected mouse brains. With respect to agglutination of hamster cells, an inhibitor of agglutination is present not only in suspensions of normal mouse brain but also in normal serum and antiserum against the neurotropic viruses.

The fact that neurotropic viruses are often used in the form of mouse brain suspensions renders it important therefore for investigators to use proper controls for the variable of the test and, in addition, to identify positive reactions by specific means.

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