TABLE II. Daily Excretion of FA and CF.

| Subject | Day | μg CF/24 hr | μg FA/24 hr |
|---|---|---|---|
| JD | 1 | .30 | 4.34 |
| | 2 | .49 | 1.00 |
| | 3 | .64 | 2.04 |
| KI | 1 | .30 | .30 |
| | 2 | .35 | .13 |
| | 3 | .41 | 1.06 |
| SM | 1 | .37 | .12 |
| | 2 | .40 | .66 |
| HA | 1 | .39 | .16 |
| | 2 | .47 | 1.11 |
| JH | 1 | .67 | 3.01 |
| | 2 | .92 | 5.58 |
| ME | 1 | 1.22 | 1.00 |
| | 2 | .57 | .72 |
| MC | 1 | 1.29 | 4.72 |
| | 2 | 1.33 | .91 |

of 1.17 and a standard deviation of ± 1.92; while CF excretion ranged from 0.19–1.6 μg per 24 hr with a mean of 0.59 and a standard deviation of ± 0.47. These results are comparable to those in other studies(4-7). The results of 24 hour urinary excretion of FA and CF in 7 subjects studied on consecutive days are given in Table II. Variation in FA and CF excretion from day to day may be a result of a varying dietary intake. In general, FA excretion varied from day to day and from subject to subject much more widely than CF excretion.

It has been reported that the bulk of CF activity in urine is in an oxygen-labile form which is degraded rapidly in air(11). No attempt was made in this investigation to protect these forms from oxidation, and the results reported here are representative only of oxygen-stable FA and CF.

*Summary.* The 24 hour urinary excretion of folic (FA) and folinic acid (CF) was determined in 51 healthy adults on unrestricted diets, using pad-plate microbiological assay. The 24 hour excretion of FA ranged from undetectable levels to 5.58 μg/24 hours with a mean of 1.17 and a stand. dev. of ± 1.92; while 24 hr excretion of CF ranged from 0.19-1.6 μg/24 hr with mean of 0.59 and a stand. dev. of ± 0.47.

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## Isolation of a Virus Closely Related to Powassan Virus from *Dermacentor andersoni* Collected along North Cache la Poudre River, Colo. (25836)

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In Sept. 1958, a 5-year-old boy from Powassan, Ontario, died of an acute encephalitis, and McLean and Donohue(1) isolated a virus from his brain. The agent, which was shown to belong to Casals' group B arthropod-borne viruses and to be related to Russian spring-summer encephalitis (RSSE) virus, was called Powassan virus. Viruses of the RSSE complex, which cause serious human illness characterized by central nervous system and

hemorrhagic manifestations, had been found previously only in Europe and Asia. During May 1952, 100 *Dermacentor andersoni* were collected along the North Cache la Poudre River, Colorado. A virus was isolated at the Rocky Mountain Laboratory from a pool of 20 of these ticks. The agent proved not to be Colorado tick fever (CTF) virus. Recently it has been shown to be closely related to Powassan virus. This paper reports data concerning isolation and serological characterization of this agent.

*Materials and methods.* In this laboratory isolations of viral agents from arthropods are carried out routinely according to the method of Eklund *et al.*(2). Antigens are prepared by extraction of suspensions of brains of infected suckling mice with acetone-ether according to the method of Clarke and Casals (3). Sera were prepared in adult mice by intraperitoneal (IP) inoculation on days 0 and 2 of 0.2 ml formalin-treated vaccine. Such vaccines consisted of a 10% suspension of brain tissue of infected suckling mice in 0.85% saline with formaldehyde added to give a final concentration of 0.5% formalin. On days 10, 15, 20, and 50 the animals were given 0.2 ml of a 10% suspension of infected brain by the same route. Mice were exsanguinated 7 to 10 days following last injection, and the serum obtained was designated as hyper-hyper-immune (HHI). HHI hamster serum was prepared by subcutaneous (SC) inoculation of adult hamsters with 0.03 ml of a $10^{-2}$ suspension of brains of infected suckling mice. Two additional SC inoculations of a $10^{-1}$ suspension of the same material were administered at 2-week intervals. The hamsters were bled 2 weeks after last inoculation. Hemagglutination (HA) and hemagglutination-inhibition (HAI) tests were done by the technics of Clarke and Casals(3), using erythrocytes from <1-day-old chicks. Sera were treated with kaolin to remove nonspecific inhibitors. For complement-fixation (CF) tests, all sera were inactivated at 60°C for 20 minutes. The next to highest dilution of complement showing complete hemolysis was used in the CF tests as 2 exact units. Antigen-serum mixtures were incubated overnight at 4°C before the hemolytic system was added. Virus neutralizations were performed with equal amounts of standard HHI mouse sera and serial 10-fold dilutions of virus incubated 1 hour in a 37°C water bath. Four-day-old Swiss albino mice (RML strain) were inoculated intracerebrally (IC) with 0.02 ml of this material and 21-day-old mice with 0.03 ml; by the IP route it was 0.05 ml for mice of either age. The Reed-Muench method was used to calculate $LD_{50}$(4).

*Results. Isolation.* The pool of 20 ticks, designated as No. 791, was triturated in 2 ml of 10% filtered normal rabbit serum in saline (FNRS-S). Each of a litter of six 4-day-old mice was inoculated IP with 0.05 ml of the supernatant of this suspension. For 7 days no signs were observed, and 3 of the mice were sacrificed. A suspension of their brains was inoculated IP into another litter. On the 8th day, one of the 3 remaining mice of the original litter was sick and on the 9th day, 2 were dead and one was sick. The last mouse died on the 13th day. On the 5th day, mice of the blind passage were sick, and in subsequent passages, incubation period has remained at 5 days. The isolate from this blind passage is designated as 791A-52 virus. Reisolation of virus from the original tick suspension was not possible, probably because the suspension had been left at room temperature for 1 day. When this agent was isolated, the only viruses being handled in the laboratory were those of CTF, yellow fever (YF) and St. Louis encephalitis (SLE). The isolate was shown not to be any of these 3 viruses. Since this virus had not been encountered previously in the laboratory, the isolation was considered authentic.

*Serological characterization. Hemagglutination.* Optimal conditions for demonstrating HA were pH 6.8 and 22°C. However, HA occurred at a pH range of 6.0 to 7.0 and at temperatures of 4°C, 22°C and 37°C. A rough HA pattern was exhibited at 4°C and pH 6.0; under all other conditions, a smooth HA pattern was observed.

Antigens prepared in the same manner from RSSE (Absettarov strain) and Powassan viruses were also tested for HA properties (Ta-

TABLE I. Hemagglutination Titers of Antigens Prepared from Brain Tissue of Suckling Mice Infected with Viruses of 791A-52, RSSE (Absettarov Strain) or Powassan.

| Antigen | Temp., °C | pH 6.0 | 6.2 | 6.4 | 6.5 | 6.6 | 6.8 | 7.0 | 7.2 |
|---|---|---|---|---|---|---|---|---|---|
| 791A-52 | 4 | 640* | 10240>† | 10240> | | 10240> | 10240> | 5120 | |
| | 22 | 1280 | 5120 | " | | " | " | 10240> | |
| | 37 | " | 640 | 640 | | 320 | 640 | 640 | |
| RSSE (Absettarov) | 4 | 0 | 0 | 0 | | " | " | 160 | |
| | 22 | 0 | 0 | 0 | | 0 | 0 | 0 | |
| | 37 | 0 | 0 | 0 | | 0 | 0 | 0 | |
| Powassan | 4 | | | 0 | 1280 | 1280 | 0 | 0 | 0 |
| | 22 | | | 0 | 0 | 0 | 0 | 0 | 0 |
| | 37 | | | 0 | 0 | 0 | 0 | 0 | 0 |

* Signifies the reciprocal of highest dilution of antigen agglutinating < 1-day-old chick erythrocytes.

† > = or greater.

0 signifies hemagglutination titer <20.

RSSE and Powassan antigens exhibited rough agglutination; 791A-52, smooth agglutination.

ble I). A prozone was observed in the HA pattern produced by Powassan virus: hemagglutination did not occur in antigen dilutions of 1:20 and 1:40.

*Hemagglutination-inhibition.* Eight units of hemagglutinating antigen prepared from 791A-52 were tested against serial dilutions of HHI mouse sera prepared against Chikungunya, Eastern equine encephalitis, Sindbis and Western equine encephalitis viruses of group A (Casals' classification of arthropod-borne viruses), 10 viruses belonging to Group B (Table II), Bunyamwera and Wyeomyia viruses of the Bunyamwera group, miscellaneous viruses including CTF (Florio strain), lymphocytic choriomeningitis, Turlock and the mouse virus GD-7, as well as a strain of virus isolated in this laboratory from *Aedes trivittatus* and one from *Culiseta inornata.* HHI mouse sera prepared against 3, as yet unidentified, viruses, and HHI hamster sera prepared against 791A-52 virus were included in the HAI test. None of the sera except those prepared against viruses belonging to group B and the homologous virus inhibited agglutination. Serum dilutions which inhibited 8 HA units of antigen varied from 1:10 to 1:2560.

TABLE II. Inhibition of 791A-52 Hemagglutinin by Hyper-hyper-immune Mouse Serum Prepared against Various Group B Viruses.

| Sera | Titer | Sera | Titer |
|---|---|---|---|
| Jap B (K-29) | 2560* | Powassan | " |
| West Nile | " | St. Louis (Mosq. 798-55) | 160 |
| Murray Valley encephalitis | 1280 | Dengue (N. Guinea B) | " |
| Ilheus | " | Dengue (Hawaii) | 10 |
| St. Louis (Parton) | 640 | 791A-52 (HHI hamster) | 320 |
| Zika | " | | |
| Ntaya | 320 | | |

* Reciprocal of dilution of serum inhibiting 8 units of hemagglutinin.

Lowest dilution serum tested: 1/10.

*Complement-fixation.* Serial dilutions of 791A-52 antigen were tested against serial dilutions of HHI mouse sera prepared against a number of group B arthropod-borne viruses. HHI hamster serum prepared against the virus of 791A-52 was used in this study. Other group B viral antigens were tested against their homologous HHI sera as controls to demonstrate virus relationships. All of the homologous antigens were tested against other group B antisera (Table III). The antiserum prepared against Powassan virus was the only one which gave a high CF titer in presence of 791A-52 antigen. Antisera prepared against SLE (Parton strain), Japanese B (Korea-29 strain), Ilheus, Murray Valley encephalitis (MVE), and West Nile viruses showed only slight relationships to 791A-52 when tested against its antigen. Complement was not fixed when 791A-52 antigen was tested against antisera prepared against the viruses of Uganda-S, Zika, Ntaya, YF (17-D strain), or against serum obtained from normal mice.

*Neutralization.* The 791A-52 virus was neutralized by Powassan and RSSE HHI sera

to titers approximately as high as those obtained with the homologous serum (Table IV). The 791A-52 HHI hamster serum neutralized Powassan and the homologous virus to about the same titer. The RSSE virus was neutralized by 791A-52 serum to low titer only in 21-day-old mice by the IP route. In

TABLE III. C-F Tests with Group B Viral Antigens and Their Respective Hyper-hyper-immune Mouse Sera.

| Antigen | Sera: 791A-52* | Powassan | St. Louis (Parton) | Jap B (K-29) | Ilheus | MVE | West Nile | a. Uganda-S b. Zika c. Ntaya d. YF (17-D) e. Normal |
|---|---|---|---|---|---|---|---|---|
| 791A-52 | | 32/128† | 8/8 | 8/2 | 4/2 | 8/2 | 4/2 | All 0/0 |
| Homologous | 128/256>‡ | 64/256> | 128/256> | 128/128 | 256/256> | 256/256> | 256/256> | a. 128/256><br>b. "<br>c. 128/64<br>d. 64/64<br>e. 0/0 |

* Hyper-hyper-immune hamster serum—slightly anticomplementary.

† Numerator represents maximum dilution of serum fixing complement in presence of antigen; denominator, maximum dilution of antigen fixing complement in presence of antiserum. 0/0 signifies that no fixation occurred even when a 1:4 dilution of serum was mixed with the undiluted antigen.

‡ > = or greater.

TABLE IV. Cross-Neutralization Test (791A-52, Powassan and RSSE). $LD_{50}$'s and logs protection in mice.

| Virus | | Sera: FNRS 4-day IC | FNRS 4-day IP | FNRS 21-day IC | FNRS 21-day IP | 791A-52* 4-day IC | 791A-52* 4-day IP | 791A-52* 21-day IC | 791A-52* 21-day IP | Powassan 4-day IC | Powassan 4-day IP | Powassan 21-day IC | Powassan 21-day IP | RSSE 4-day IC | RSSE 4-day IP | RSSE 21-day IC | RSSE 21-day IP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 791A-52 | T† | 8.9 | 8.9>‡ | 8.4 | 7.5 | 6.2 | 4.2‖ | 5.7 | 1.5<§ | 4.8 | 2.3 | 4.7 | 1.5< | 5.7 | 6.3> | 5.7 | 2.5‖ |
| | LP† | | | | | 2.7 | 4.7> | 2.7 | 6.0> | 4.1 | 6.6> | 3.7 | 6.0> | 3.2 | 2.6± | 2.7 | 5.0 |
| Powassan | T | 9.6 | 8.5 | 9.0 | 8.0 | 6.6 | 6.0 | 6.4 | 4.1‖ | 4.8 | 1.6< | **5.0** | **1.5<** | | | | |
| | LP | | | | | 3.0 | 2.5 | 2.6 | 3.9 | 4.8 | 6.9> | 4.0 | 6.5> | | | | |
| RSSE | T | 9.3> | 7.5 | " | 8.7 | 8.3> | 6.5> | 8.4> | 5.9 | | | | | 6.4 | 3.6 | 6.3 | 1.8< |
| | LP | | | | | 1.0< | 1.0± | .6± | 2.8 | | | | | 2.9> | 3.9 | 2.7 | 6.9> |

* Hyper-hyper-immune hamster serum diluted 3 times.

† T = titer. LP = logs protection.

‡ > = or greater.

§ < = or less.

‖ Prolonged incubation period and irregular death pattern.

another experiment (unpublished data), the RSSE serum did not neutralize Powassan virus by the IC route of inoculation in 21-day-old mice. Those data indicate that the relationship between 791A-52 and Powassan viruses and RSSE virus is nonreciprocal.

*Conclusions.* A virus closely related, if not identical, to Powassan virus has been isolated from *Dermacentor andersoni* from northern Colorado. A nonreciprocal relationship between RSSE virus and 791A-52 virus is indicated by the cross neutralization tests. The importance of *Dermacentor andersoni* in maintenance of this virus is unknown. Although thousands of this tick species collected in many areas in the western United States have been examined for viruses in this laboratory, this is the only isolation of this agent. Since these prior studies of ticks at the Rocky Mountain Laboratory were directed specifically toward study of the ecology of Colorado tick fever virus, the ecology of the 791A-52 virus is unknown. Field and laboratory studies on this subject are in progress.

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Since this paper was submitted, an article has appeared by J. Casals (*Can. Med. Assn. J.*, 1960, v82, 355) showing that Powassan virus is more closely related to the RSSE complex of viruses than to any other arbor virus and is a distinct virus.

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## Connective Tissue III. Collagen and Hexosamine Content of Tissues of Rats of Different Ages.* (25837)

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Sobel and his co-workers have reported that hexosamine and collagen content of skin of rat, guinea pig, squab, rabbit and human, femur of rat and lung of rabbit(1,2,3,4) increase with age. They have also shown that rate of hexosamine deposition decreased with age more than that of collagen deposition; therefore, ratio of hexosamine to collagen decreased with age. Houck and Jacob(5) reported that concentration of hexosamine in rat skin was relatively unaffected by age of the animal, but ratio of hexosamine to nitrogen decreased with increases in body weight up to 330 g. Above this figure, however, the ratio varied directly with body weight. The present study is concerned with determination of the relation of age to: (1) total protein, (2) collagen and (3) hexosamine in aorta, tendon and skin of rat.

*Methods.* Male and female rats, Wistar strain, were used in this study. Rats from 4 weeks to 8 months of age were raised in our laboratory on Purina chow and water *ad lib.* Two-and-one-half-year-old rats were purchased from Research Supply Co., Philadelphia, Pa. One-tenth to 0.5 g of tissue were extracted with fat solvents(6). For total protein and collagen determinations the fat free tissues were hydrolyzed in 6N HCl at 110°C for 16 hours in a sealed tube. Nitrogen was determined by Conway microdiffusion method (7) and protein was calculated as 6.25 x nitrogen. Hydroxyproline was determined by the method of Neuman and Logan(8). Collagen in tendon and skin was calculated by multiplying the amount of hydroxyproline by

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