acid. Degradation studies on the ring system of this radioactive β-carotene indicate that the carboxyl carbon of acetate is incorporated into positions 1, 1′, 3, 3′, 5 and 5′ of the ring system whereas the methyl carbon is incorporated into all positions of the ring system. Our studies also indicate that the ring carbons are more radioactive than the side chain carbon atoms.

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Serum Sialic Acid Levels in Experimental Anaplasmosis.* (28702)

P. J. RAO AND MIODRAG RISTIC (Introduced by N. D. Levine)
Department of Veterinary Pathology and Hygiene, College of Veterinary Medicine, University of Illinois, Urbana

Members of the sialic acid group of compounds occur in various tissues and body fluids(2,12), viz., mucous secretions, urine, egg albumin, milk, cerebrospinal fluid, blood serum, salivary gland, and brain. Sialic acid is the group name for several compounds of substituted neuraminic acid derivatives (N-acetyl, N-glycolyl, N,O-diacetyl-neuraminic acid). The unsubstituted 9-carbon compound is called neuraminic acid(3). As may be seen from Fig. 1, N-acetyl neuraminic acid (NANA) may be visualized as an aldol condensation product of N-acetyl hexosamine and pyruvic acid(12). On deacetylation, NANA yields neuraminic acid(4). In nature, sialic acids occur as components of mucoproteins(2).

Sialidase (neuraminidase) is an enzyme present in bacteria and in myxoviruses. Its action upon receptors of tissue cells, which are mucoprotein in nature, results in liberation of sialic acid. It is believed that the activity of sialidase serves to attach the virus particles to the erythrocytes or susceptible host cells as a first step in the invasion of the latter cells(4).

Anaplasma marginale, the etiologic agent of anaplasmosis, is considered a rickettsial parasite of erythrocytes of cattle(5). Anaplasmosis is an infectious and transmissible disease of cattle manifested by progressive anemia associated with the presence of intraerythrocytic inclusions of *A. marginale* (Fig. 2). Electron microscopic and fluorescent antibody studies of *A. marginale* have provided static evidence that the initial bodies of this parasite are capable of attachment to, and penetration of, the erythrocytic membrane(6). Therefore, it was of interest to know whether the sialic acid of the mucoproteins of the receptors of erythrocytes is

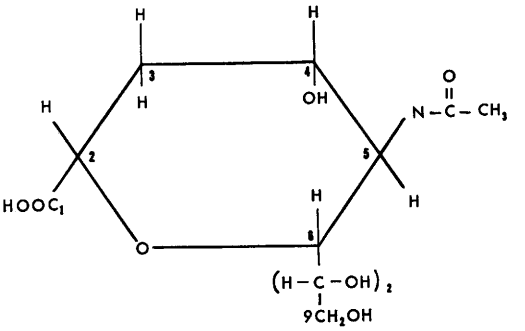

FIG. 1. Probable structure of N-acetyl neuraminic acid (after Whitehouse, M. W., Zilliken, F., *Methods of Biochemical Analysis*, v8, 199. Ref. 12.).

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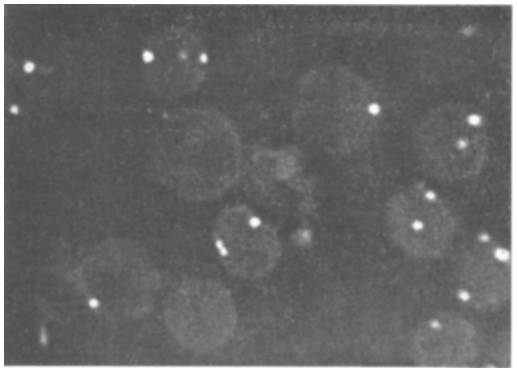

FIG. 2. *Anaplasma marginale* infected bovine erythrocytes stained with fluorescein-labeled anti-*Anaplasma* globulin and examined with ultraviolet microscope. × 550.

involved in the attachment of the *Anaplasma* initial bodies to the erythrocytes. It was postulated that, if the *Anaplasma* initial bodies have sialidase enzyme, it might be possible to detect an increase of free sialic acid in the blood serum during acute anaplasmosis infection. With this in mind, serum sialic acid levels were determined in an effort to secure indirect evidence that the sialic acid of the mucoproteins of the erythrocytic receptors play a role in the attachment of *Anaplasma* initial bodies. Reports that there is an increase in serum sialic acid in certain bacterial and viral diseases, viz., tuberculosis(8) and influenza(9) have promoted wide interest in the study of serum sialic acid fluctuations in health and disease. Therefore, it was deemed desirable to study the sialic acid content of the serum in a rickettsial disease such as *Anaplasmosis*.

Materials and methods. Calves used in the experiments were of mixed breeds and between 4 months and 1 year of age. Blood from calves acutely infected with *Anaplasma* or which were carriers of the agent was inoculated in the calves by the subcutaneous or intravenous route, or by both routes. Blood smears were stained with acridine orange and examined under the fluorescent microscope(11) to determine the percentages of infected erythrocytes containing large *Anaplasma* organisms. Serum for analysis was collected from the blood clot following incubation for 1 hour at 37°C. The serum was clarified by centrifugation and stored at −10°C until all samples from a given calf had been collected. Free sialic acid was estimated by precipitating 1.0 ml of serum or a 1:2 dilution of it in physiological salt solution with 1.0 ml of 10% trichloroacetic acid (TCA). The precipitate was centrifuged at 2000 × *g* for 10 minutes and the supernatant fluid was used for estimating sialic acid as described below.

For estimation of total sialic acid, serum samples were hydrolyzed by 2 different methods. In one method(10), 1.0 ml of serum was precipitated with 1.0 ml of 10% TCA, heated for 5 minutes in boiling water, cooled under tap water and centrifuged at 2000 × *g* for 10 minutes. The clear supernatant fluid was used for sialic acid assay. In the second method(1), 1.0 ml of a 1:10 dilution of serum in physiologic salt solution in triplicate tubes was precipitated with 1.0 ml of 10% TCA; the mixture was heated in a water bath at 80°C for 1 hour during which time the tubes were agitated twice. After cooling with tap water, the tubes were centrifuged and the supernatant fluid was assayed for sialic acid. It was determined in a separate experiment that the hydrolysis by the first method yielded 2.15 times less sialic acid than by the second method. Therefore, values obtained were multiplied by 2.15. Nevertheless, only one of the 2 hydrolytic methods was used throughout the experiments on any particular calf.

Sialic acid was estimated by the method of Warren(2), except that 0.4 ml instead of 0.2 ml of the standard solution or the solutions suspected to contain sialic acid were used. Triplicate samples were used in most cases, except that duplicate samples served in a few cases where the variation among these replicate tests was negligible. A molar extinction coefficient of 57,000 was used to calculate the concentrations of sialic acid(1,2, 11)†.

Results. Fig. 3 shows that the peak of absorption spectrum of the colored product (chromophore) which developed with known crystalline NANA was identical with the

† We are grateful to Dr. L. J. Warren, Nat. Inst. Arthritis and Metab. Dis., Nat. Inst. Health, U.S.P.H.S. for a gift of N-acetyl neuraminic acid.

TABLE I. Free and Total Sialic Acid Contents of Serum of Calves Infected with *A. marginale.*

Calf	Dose of inoculum and route	Sialic acid†	Pre-infection content (μg/1.0 ml serum) Range	Mean ± S.E.*	Post-infection content (μg/1.0 ml serum) Range	Mean ± S.E.*	P	% increase of max sialic acid contents before and after infection	% erythrocytes infected at time of max increase of sialic acid	Max % of infected erythrocytes during disease
77 (splenectomized)	100 ml, I.V.,* 120 ml, S.C. carrier blood	Total	610–667 (4)	630 ± 14.11	723–944 (10)	843 ± 24.51	<.0005	41	24	39
		Free	11.4–16.3 (4)	14.3 ± 1.03	11.3–40.8 (11)	22.7 ± 3.40	<.1	150	nil	
59 (splenectomized)	5.0 ml, I.V., acute blood	Total	859–882 (3)	872 ± 6.57	890–1021 (4)	985 ± 18.34	<.025	15	nil	100
		Free	11.3–13.8 (3)	12.2 ± 1.82	11.5–32.8 (5)	27.2 ± 3.99	<.0005	137	11	
75 (non-splenectomized)	250 ml, I.V., carrier blood	Total	573–731 (9)	659 ± 19.64	641–777 (13)	713 ± 12.19	<.0025	6.3	7	16
		Free	5.4–9.2 (8)	6.8 ± .45	6.0–11.5 (14)	8.6 ± .43	<.01	25	5	
65 (non-splenectomized)	5.0 ml, I.V., acute blood	Total	594–719 (4)	651 ± 30.44	534–888 (17)	678 ± 23.37	<.0005	23	21	21
		Free	19.2–21.8 (3)	20.36 ± .75	7.3–31.8 (21)	20.0 ± 1.36	n.s.	46	8	
39 (non-splenectomized)	5.0 ml, I.V., acute blood	Total	327 (2)	327 ± 0	308–389 (3)	341 ± 14.26	n.s.	19	62	100
20 (non-splenectomized)	1000 ml, I.V., acute blood	Total	257–270 (3)	266 ± 7.17	264–371 (3)	312 ± 18.05	<.01	37	8	12

* S.E. = Standard error; I.V. = Intravenous; S.C. = Subcutaneous; P = Probability; n.s. = Not significant.
† Total or free sialic acid.
The numbers in parentheses given under ranges indicate the No. of determinations.

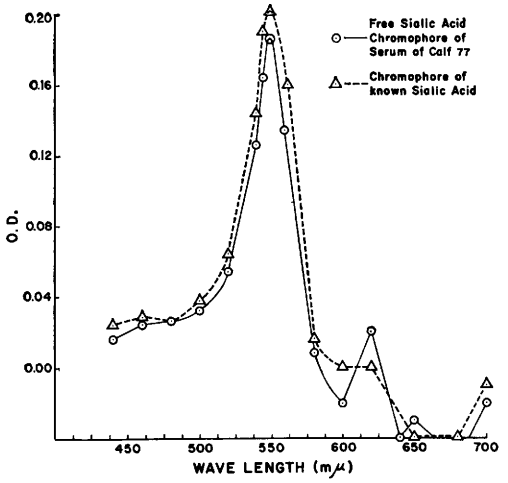

FIG. 3. A comparison of absorption spectra of known crystalline N-acetyl neuraminic acid and that extracted from the serum of calf 77 infected with *Anaplasma marginale*.

peak of absorption spectrum of the chromophore developed by the TCA extract of free serum sialic acid of the *Anaplasma* infected calf 77. Experiments on 6 calves indicated that the increases in total serum sialic acid concentration of *Anaplasma*-infected calves ranged from 6.3 to 41.0% (Table I). The results of studies on free sialic acid in 4 calves are presented in Table I. The increases of free sialic acid in the 4 calves ranged from 25.0 to 150.0%. In 2 splenectomized calves (77 and 59) the percentage increases of maximal free sialic acid before and after infection were 150.0 and 137.0, respectively, whereas the percentage increases of free sialic acid in 2 nonsplenectomized calves (75 and 65) were 25.0 and 46.0, respectively (Table I). The increase of free sialic acid was more marked (ranging from 25.0 to 150.0% in 4 calves) than the increase of total sialic acid (ranging from 6.3 to 41.0% in 6 calves). The extent of increase of free sialic acid was approximately the same regardless of the quantity of inoculum of infected blood (calves 77 and 59, Table I).

Detailed data from experiments on 2 splenectomized calves which showed an increase of free and total sialic acid in relation to the percentages of infected erythrocytes are graphically represented (Fig. 4). Noteworthy is the fact that the sialic acid increased very early in infection. The observation was substantiated by the results obtained with other calves. At the time sialic acid increased in the serum of each calf, the percentage of infected erythrocytes was low, ranging from 0 to 24% in 5 out of 6 calves investigated (see column 10 of Table I). In calf 59, the elevated serum sialic acid levels were maintained at high levels throughout the infection. In this calf, there was a rapid progressive increase in infection of erythrocytes, reaching 100% in a period of 16 days (Fig. 4). In contrast, the free sialic acid content in calf 77 was high only intermittently. In this calf, the red cell infection was low, reaching a maximum of only 39%, and the dis-

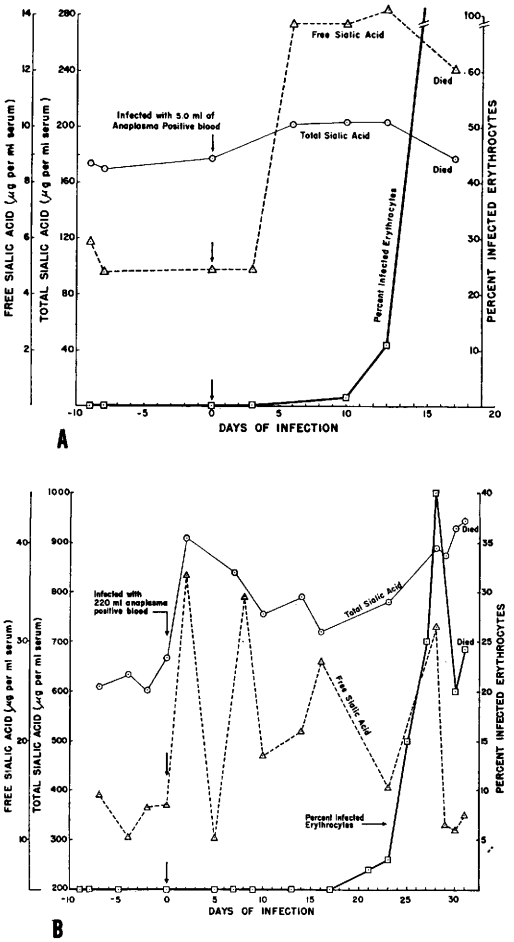

FIG. 4. Free and total serum sialic acid levels, and percentage of infected erythrocytes of calves 59 (a) and 77 (b) respectively before and after infection with *Anaplasma marginale*.

ease was prolonged over 31 days (Fig. 4).

Discussion. According to Gottschalk(13), 90% of the serum sialic acid is bound to the globulins. Therefore, increase in the concentration of globulins which occurs as a result of various immunogenic stimulation might be followed by simultaneous increases in the serum sialic acid. In our experiments, both protein bound and free sialic acid were measured. Only traces of free sialic acid were detected in preinfection serums. Warren also (2) failed to detect free sialic acid in human serum. The free sialic acid content of the serum tested by us was negligible before infection; it rose significantly shortly after infection. It is evident from Fig. 4 that there is definite increase in the free sialic acid content of serum of calf 77 soon after infection; but the statistical analysis showed only moderate significance ($P<0.1$) due to the high fluctuations of the free sialic acid content of serum during the post-infective phase. It can be noticed that the high levels of free sialic acid occurred only after infection. The best way would have been to run parallel determinations on a control calf. Such a procedure would have given a statistically significant result which is masked in this experiment. Increase of free sialic acid occurred within 2 days after infection of calf 77. This animal was inoculated with a large quantity (220 ml) of blood. Free sialic acid increased 3 days after infection of calf 59. It was inoculated with only 5.0 ml of blood. This may indicate an inverse relationship between the dose of infective material and the time lapse before an increase of free sialic acid in serum may be observed.

The fact that a relatively small quantity of infective blood stimulated an increase of free serum sialic acid suggests that the increase of free sialic acid is due to damage done by the multiplying agent rather than to the stress, if any, caused by injection of homologous blood. The fact that the more susceptible, infected splenectomized calves showed considerably higher quantities of free serum sialic acid than did nonsplenectomized infected calves is further evidence that the release of free sialic acid in serum resulted from an infectious process. Since protein bound sialic acid is found in the globulin fractions(13) of the serum, and since these fractions might be expected to increase in anaplasmosis(14), the total sialic acid increase may have resulted from both an increase of free sialic acid and of serum globulins.

The coincidence of the increased quantities of serum sialic acid with the early phase of invasion of the erythrocytes by *A. marginale* suggests that free sialic acid might have been liberated from the receptor sites of the erythrocytes by the enzymatic action of the *A. marginale* initial bodies. Direct proof of this hypothesis would require the demonstration of *in vitro* liberation of free sialic acid from substrates such as neuraminlactose or mucoproteins by the enzymatic action of a purified preparation of *A. marginale*. Research in this direction is in progress.

The fact that increase of sialic acid occurred very early during the initial phase of anaplasmosis is in agreement with the hypothesis advanced by Ristic(5) concerning the development and multiplication of the initial *Anaplasma* bodies. This assumption was based upon the sequences of electron microscopic and fluorescent antibody studies of Ristic(5) and Ristic and Watrach(6). From these studies, they conceive 4 developmental stages of the organism. The early stage is manifested by invasion of erythrocytes by initial *Anaplasma* bodies. The invasive mechanism of the organism was found to involve penetration of the erythrocytic membrane by initial bodies. It was hypothesized that a complete developmental cycle of the organism occurred in the mature erythrocytes and that binary fission of the initial body and its direct transfer between erythrocytes could explain the mode of development of the agent.

Desialization of mucoproteins results in a greatly enhanced antigenicity of these antigenically poor complexes(15). Knowing that *Anaplasma* possesses the ability to desialize erythrocytic mucoproteins may help explain one of the mechanisms of the autoimmune processes in anaplasmosis(7).

Summary. The quantities of protein-bound and free serum sialic acid of calves experi-

mentally infected with *Anaplasma,* a rickettsial parasite of erythrocytes of cattle, were ascertained at various phases of experimental infections. The percentage increase of free sialic acid in the sera of 4 calves, 2 of which were splenectomized, ranged between 25 and 150 while the bound sialic acid ranged from 6.3 and 41. In 2 splenectomized calves, the percentage increases of free sialic acid were 150 and 137 and the percentage increases of bound sialic acid were 41 and 15 respectively. In contrast, the percentage increases of free sialic acid in 2 nonsplenectomized calves were 25 and 46 and percentages of bound sialic acid in the same calves were 6.3 and 23 respectively. The percentage increase in free sialic acid was approximately the same regardless of the quantity of inoculum of infected blood, *i.e.,* 5.0 ml or 220.0 ml, used. The maximal increase in free sialic acid occurred during the early invasive stage of the disease. The possibility that the free sialic acid in serum might have originated from the receptor substances of the erythrocytes is discussed in the light of a hypothesis concerning the cycle of development of the causative agent, according to which the invasive mechanism of *Anaplasma* involves penetration of the erythrocytic membrane by initial bodies of this organism.

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Induced Resistance to Electroshock and Audiogenic Seizures in Inbred Strains of Mice.* (28703)

MAX HAMBURGH AND WALTER B. ESSMAN†
(Introduced by William Etkin)
Department of Anatomy and Department of Rehabilitation Medicine, Albert Einstein College of Medicine, New York City

Attempts to identify physiological mechanisms and the sites of neurological lesions implicated in genetic audiogenic seizures in rodents have largely employed two methods: (1) Tests for the effectiveness of various biochemical compounds and drugs that change the characteristic seizure pattern in susceptible animals; (2) Ablation experiments to analyze the effect of discrete neuroanatomical lesions on seizure pattern and incidence in rodents genetically predisposed to audiogenic convulsions.

The results of these studies have implicated metabolic intermediates(1) steroid hormones(2) and several depressant, sedative and analgesic drugs(3). Extensive ablation of cortical and some subcortical structures re-

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† Present address: Dept. of Psychology, Queens College, N. Y.