

INHIBITION OF THEILER'S ENCEPHALOMYELITIS VIRUS
(GDVII STRAIN) OF MICE BY AN INTESTINAL
MUCOPOLYSACCHARIDE

II. PURIFICATION AND PROPERTIES OF THE MUCOPOLYSACCHARIDE*

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In the preceding paper (1) it was shown that the intestinal tissue of adult mice contains a substance capable of inhibiting the GDVII strain of Theiler's encephalomyelitis virus of mice. The inhibitor was demonstrated to be active against the hemagglutinating, as well as the pathogenic activities of the virus. Evidence was presented which showed that the inhibitor is highly specific in its action, and that it functions by combining with the virus.

In the present paper the method of purification of the inhibitor will be described. The results of studies of some of the physicochemical properties which indicate that the inhibitor is a mucopolysaccharide will be presented.

Materials and Methods

Acetone-Dried Powder.—The preparation of an acetone-dried powder from tissue and the methods for measuring inhibitory activity are described in the preceding paper (1).

Chromatographic Analyses.—The identification of amino acids was carried out following the method of Consden, Gordon, and Martin (2). Two dimensional analysis was carried out using Whatman 1 paper with water-saturated *s*-collidine and water-saturated phenol as the solvents. With the latter solvent, the aqueous phase in the tank contained 3 per cent NH₃ and a trace of KCN was added as described (2). A tank was constructed following the directions of Toenies and Kolb (3). The spraying reagent was a 0.1 per cent solution of ninhydrin in butanol containing 1 per cent acetic acid (4).

EXPERIMENTAL

Purification of the GDVII Virus Inhibitor.—A large pool of acetone-dried powders of mouse intestine was extracted with 10 volumes of distilled water at 37°C. for 1 hour. After centrifuga-

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tion at 2500 R.P.M. for 30 minutes, the supernate was decanted and saved. The sediment was washed once with a small volume of water, the washings combined with the supernate, and the sediment discarded. Advantage was taken of the fact that this crude extract contains phosphatase. Magnesium chloride was added to a concentration of 0.005 M, the pH adjusted to 8, and the extract was incubated at 37°C. At the start of incubation the total phosphorus content was determined on a sample. After 2 to 3 hours' incubation nearly all the organic phosphorus was liberated as inorganic phosphorus.

To each liter of solution, 250 ml. of 25 per cent trichloroacetic acid was added. After centrifugation at 2500 R.P.M. for 30 minutes, the supernatant solution was decanted and saved. The sediment was washed with a small volume of 5 per cent trichloroacetic acid and the washings were added to the supernatant solution which was then dialyzed against distilled water in the cold for 24 hours with several changes of water. During dialysis a fine precipitate which developed was removed by centrifugation. In order to remove any residual protein from this solution, 200 ml. of chloroform and 40 ml. of *n*-butanol were added to each liter and the mixture shaken vigorously for 30 minutes in a shaking machine. After centrifugation, the

TABLE I
Purification of the GDVII Virus Inhibitor from Intestinal Tissue of Adult Mice

| Fraction | Weight | Volume of extract | Total HIU* | Specific activity | Yield | Purification |
|--|------------|-------------------|----------------|-------------------|-----------------|--------------|
| | <i>gm.</i> | <i>ml.</i> | <i>million</i> | <i>HIU/gm.</i> | <i>per cent</i> | |
| Acetone-dried powder | 144.0 | 1410 | 38.5 | 0.27 | — | — |
| TCA† supernate (dialyzed) | — | 1970 | 26.8 | — | 70 | — |
| After chloroform treatment | — | 1960 | 26.7 | — | 70 | — |
| Alcohol precipitate | 1.507 | 24.7 | 16.8 | 11.2 | 44 | 41 |
| CuSO ₄ supernate (dialyzed) | — | 42.0 | 7.2 | — | 19 | — |
| Final | 0.2003 | — | 6.2 | 31.0 | 16 | 115 |

* Hemagglutination-inhibition units.

† Trichloroacetic acid.

upper aqueous phase was siphoned off, fresh chloroform and butanol added to it, and the shaking was repeated. After 3 or 4 cycles, a precipitate no longer formed at the interface.

To each liter of the deproteinized solution, 100 gm. of sodium acetate (NaC₂H₃O₂·3H₂O) was added. The polysaccharide was then precipitated by adding 3 volumes of 95 per cent ethanol and allowing the mixture to stand in the cold overnight. The precipitate was collected by centrifugation, washed successively with small volumes of absolute ethanol and ether, and dried *in vacuo*. The inhibitor was extracted from this powder with distilled water. Residual nucleic acid was removed from this extract by adding 1 volume of 10 per cent CuSO₄·5H₂O to each 5 volumes of solution and adjusting the pH to 5.1. The resulting precipitate was removed by centrifugation, washed with a small volume of 2 per cent CuSO₄·5H₂O, and discarded. The combined supernatant solutions were dialyzed against several changes of 0.01 N HCl for 24 hours and then against several changes of distilled water for 24 hours. After dialysis the solution was lyophilized.

In order to determine purification and yield, aliquots were collected at each step and inhibitory activity was measured by hemagglutination inhibition.

A protocol for one preparation is shown in Table I. Specific activity is expressed as hemagglutination-inhibition units per gram of dry weight. The ac-

tivity was determined on an *extract* of the dry powder in the case of the acetone powder and the alcohol precipitate. The final lyophilized material is completely soluble. The data in Table I show that a 115-fold purification was achieved with a yield of 16 per cent.

The purification method of Morgan and King (5), which has proved to be highly effective with the blood group substances, was tried for the purification of the GDVII virus inhibitor. Although a relatively pure product was obtained, the yield was only about 10 per cent of the amount obtained by the above described procedure.

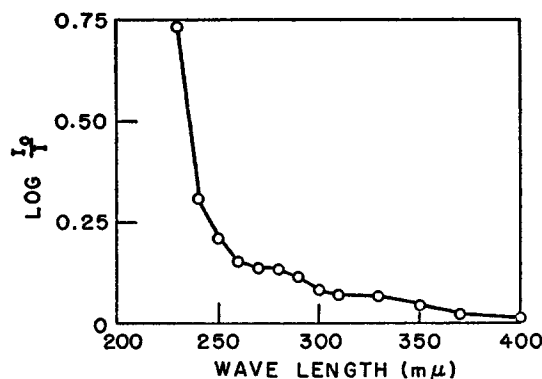


FIG. 1. Ultraviolet absorption spectrum of the inhibitor (1.0 mg./ml. in distilled water) measured in a 1 cm. cell with a Beckman model DU spectrophotometer.

Physical Properties.—A 1 per cent solution of the inhibitor in water is clear with a barely perceptible brownish color. The inhibitor in neutral solution is stable to prolonged storage in the frozen state, and may be heated for 10 minutes at 115°C. without adverse effect. When a sample of the dry powder was heated at 100–105°C. for 3 days in an oven, the activity was reduced to 25 per cent of its original value.

The viscosity of a 1 per cent solution in distilled water was determined in an Ostwald viscosimeter at 37°C. and was found to be 2.31, relative to water.

The absorption spectrum of a 0.1 per cent solution, determined in a 1 cm. cell in a Beckman spectrophotometer, is shown in Fig. 1.

Water Content.—The water content of the powder obtained after lyophilization was determined by heating a sample at 100–105°C. to constant weight; it was found to contain 15 per cent water. All subsequent analytical data will be reported as corrected values.

Phosphorus and Nitrogen.—The total phosphorus content was found (6) to be 0.08 per cent. The maximum amount of nucleic acid that could be present therefore is about 1 per cent. The nitrogen content was found to be 5.9 per cent by direct nesslerization as described by Koch and McMeekin (7).

Carbohydrate Components.—The rate of appearance of reducing sugars and hexosamine was determined by heating 1 mg. samples of the inhibitor with 0.5 N HCl at 100°C. After varying periods of time the tubes were removed and opened. 90 per cent of the amount of sodium hydroxide required to just neutralize the hydrolysis mixture was added cautiously. Aliquots of each hydrolyzed sample were used for the determination of reducing sugars (8) and hexosamine (9). The results are depicted graphically in Fig. 2.

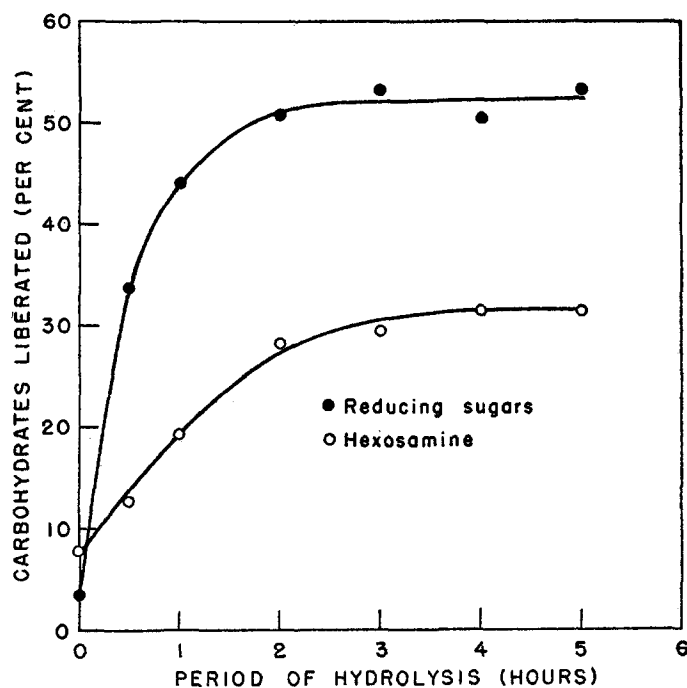


FIG. 2. Hydrolysis of inhibitor in 0.5 N HCl at 100°C. with liberation of reducing sugars (as glucose) and hexosamine (as glucosamine).

As shown in Fig. 2, the 2 curves reached a maximum at about 3 hours at which time 53.4 per cent of the original dry weight was present as reducing sugars and 31.8 per cent as hexosamine. An analysis of the unhydrolyzed inhibitor showed 3.7 and 6.5 per cent reducing sugars and hexosamine respectively.

N-Acetylhexosamine and methylpentose were determined by hydrolyzing 1 mg. samples of inhibitor in 2 N acetic acid at 100°C. in sealed tubes. After various periods of heating, the tubes were opened and the contents evaporated to dryness in a vacuum desiccator containing sulfuric acid and potassium hydroxide. The residues were redissolved and each hydrolysate was analyzed for

N-acetylhexosamine (10), methylpentose (11), reducing sugars, and inhibitory activity.

As shown in Fig. 3, *N*-acetylhexosamine reached a maximum of 20 per cent after 7 hours. A control (*N*-acetylglucosamine) treated as above showed no decrease after 7 hours, but after 24 hours only 90 per cent of the theoretical amount was recovered. Total reducing sugar content had not reached a maxi-

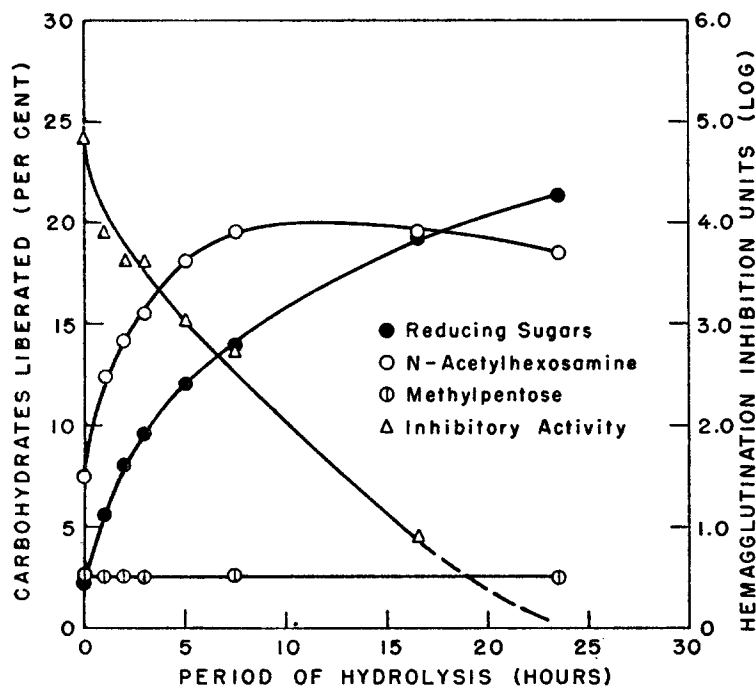


FIG. 3. Hydrolysis of inhibitor in 2 N acetic acid at 100°C. with liberation of reducing sugars (as glucose), *N*-acetylhexosamine (as *N*-acetylglucosamine), and methylpentose (as fucose). Destruction of biological activity is shown by the decrease in hemagglutination inhibition units (logarithmic scale) with time of hydrolysis.

mum even after 24 hours whereas methylpentose showed no increase over the value obtained for the unhydrolyzed material which was 2.7 per cent. Inhibitory activity diminished rapidly, about 10 per cent remaining after the 1st hour of heating.

By means of the cysteine reaction (SCyRI) described by Dische *et al.* (12), the presence of galactose was demonstrated. An analysis for hexuronic acid by the carbazole reaction (13) revealed that 8.3 per cent, as glucuronic acid, was present.

When total carbohydrate content was determined by a modification of the

anthrone reaction (14), the value obtained was 21.9 per cent as glucose, which is considerably lower than the value of 53.4 per cent obtained by determining the reducing sugars on a hydrolyzed specimen. An investigation of the specificity of the anthrone reaction showed that neither hexosamine nor hexuronic acid reacted with anthrone to produce the characteristic color. When the values of these 2 carbohydrates are added to that obtained with the anthrone reagent, there is an approximate agreement with the result obtained for reducing sugars after hydrolysis.

Amino Acid Components.—The "protein" content of the inhibitor was found to be 17.5 per cent by the method of Lowry *et al.*, (15) with a purified casein standard. A spectrophotometric assay (16) showed 11.2 per cent "protein." In view of the drastic deproteinizing measures employed in the purification procedure, it seems unlikely that any native protein as such is present. Furthermore, since both methods are actually based upon the presence of certain amino acids, it seems preferable at present to view the above results as indicating the presence of peptides or amino acids combined with the polysaccharide. On this basis the inhibitor has been considered to be a mucopolysaccharide.

The identification of the individual amino acids by partition chromatography was undertaken.

A sample of the inhibitor was hydrolyzed for 18 hours with 6 N HCl at 110°C. in a sealed glass tube. The hydrolysate had become dark amber in color and contained a black precipitate. The precipitate was removed by centrifugation and washed. The washings and supernatant fluid were combined and evaporated to dryness *in vacuo*. The residue was dissolved in a small volume of water and analyzed for amino acids by two dimensional chromatography.

The hydrolysate of the inhibitor was resolved into 7 spots. One, with an R_f of 0.08 in phenol, could not be identified. The remaining spots corresponded in their positions to those of aspartic acid, glutamic acid, serine, threonine, alanine, and isoleucine.

Periodate Oxidation.—It has been shown that the receptor substances for the influenza-mumps-Newcastle disease virus group are inactivated by periodate (17). Similarly, the mucopolysaccharide blood group substances have been shown (18) to be oxidized by periodate. The stability of the GDVII virus inhibitor towards this reagent was investigated.

To 0.3 ml. of an inhibitor solution (1 mg./ml.), 0.15 ml. of 0.1 M lithium periodate was added and the mixture was held for 23 hours at room temperature. The reaction was stopped by adding 0.05 ml. of 50 per cent glucose. As a control, the periodate and glucose were mixed 3 hours prior to the addition of the inhibitor. The mixture was then held for 23 hours at room temperature. Each mixture was assayed for inhibitory activity *in vivo* and *in vitro*. As shown in Table II, destruction of the inhibitor by periodate was almost complete.

Homogeneity of the Purified Inhibitor.—From the preceding data it is clear that the most highly purified inhibitor preparation contains carbohydrate and

amino acid components. Since data are not available regarding the homogeneity of the preparation, the question arises regarding the nature of the biologically active material. The possibilities to be considered are (a) either the carbohydrate or the amino acid component is the active substance; (b) the biological activity is associated with both components; (c) the active substance is present as a third component. This problem was approached by attempting to separate the above components by partition chromatography and determining biological activity.

TABLE II
Effect of Lithium Periodate on the Activity of the GDVII Virus Inhibitor

| Specimen | Hemagglutination inhibition | Infectivity inhibition* $\frac{1}{T}$ | Virus control $\frac{1}{T}$ |
|-----------------------|-----------------------------|--|--------------------------------|
| | <i>HIU/ml.</i> | | |
| Inhibitor + periodate | 100 | 0.24 | 0.26 |
| Control | 3,200 | 0.03 | |

* Each specimen was diluted 1:4 before it was mixed with an equal volume of the virus suspension; consequently there were 12.5 and 400 HIU per ml. respectively in the inocula.

Six sheets of Whatman 1 paper, 40 by 4 cm. were washed with the solvent to be used. When dry, each sheet was marked off at the edges at 1 cm. intervals. On each of three of the sheets a solution containing 2 mg. of inhibitor was placed 4.5 cm. from the lower edge. The remaining 3 sheets served as controls. Each pair of sheets was suspended in a chromatography chamber with about 1 cm. of the lower edge immersed in the developing solvent. The following solvents were employed: (a) 0.1 M phosphate buffer, pH 7.3; (b) 55 per cent ethanol; (c) 60 per cent ethanol. When the solvents had ascended approximately 30 cm., the sheets were removed, dried at room temperature, and cut into 1 cm. strips. Each strip was placed in a test tube containing 1.5 ml. water, the tubes were heated for 1 hour at 60°C. and the paper strips were removed. The eluates were centrifuged and the supernatant fluids were pipetted off, care being taken to avoid carrying over any paper fibers. Each eluate was analyzed for (a) carbohydrate by the SCyRI reaction of Dische (12) using galactose as a standard; (b) "protein" by the method of Lowry *et al.* (15) with a purified casein standard; (c) inhibitory activity by the hemagglutination-inhibition method. The values obtained for the control sheets were used as blank values and the experimental results were corrected accordingly.

The results of these experiments are shown in Figs. 4 to 6. It can be seen that the inhibitor migrated most rapidly when the solvent was phosphate buffer (Fig. 4), and least rapidly with the 60 per cent ethanol solvent (Fig. 6). Each attempt to separate the carbohydrate and "protein" components by the use of a different solvent was unsuccessful, and in each case there is an approximate parallelism between the 2 components and biological activity. The inability to separate the 2 components from each other or from the biologically active substance strongly indicates that the carbohydrate and "protein" are combined and that this complex possesses the inhibitory activity. With the ethanol

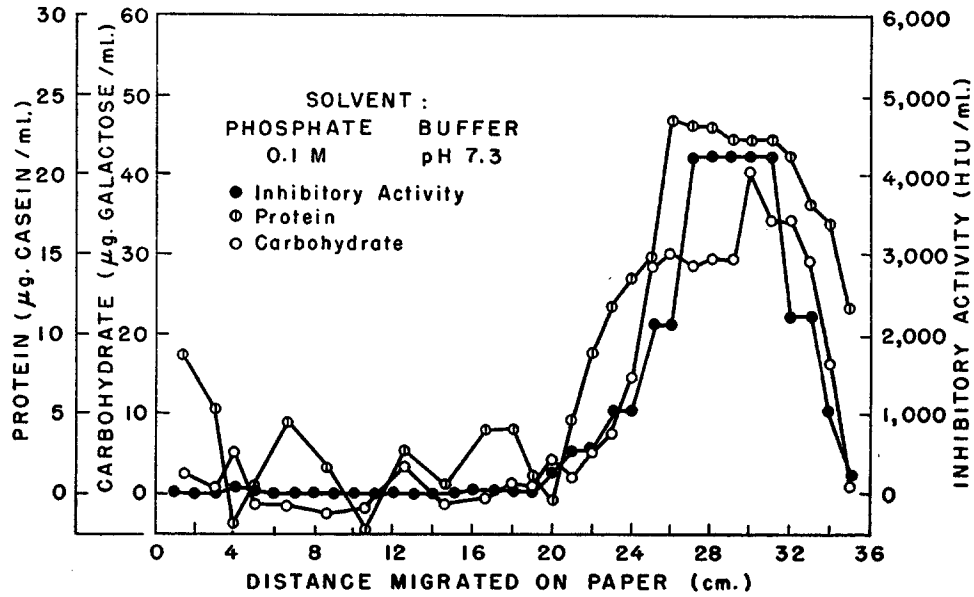


FIG. 4

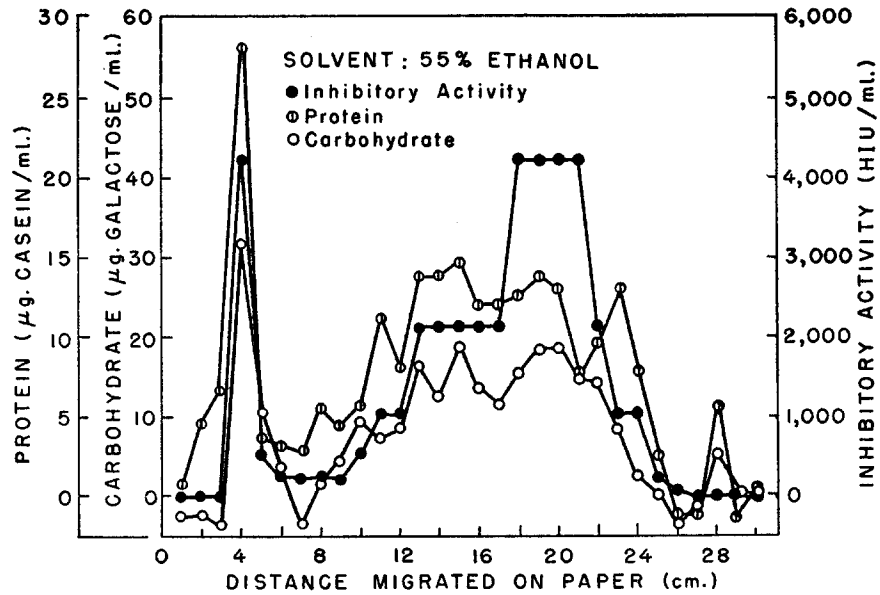


FIG. 5

FIGS. 4, 5, and 6. Partition chromatography of inhibitor on paper and analysis of eluates of different levels of the paper for inhibitory activity, carbohydrate, and "protein." Solvents used were phosphate buffer (Fig. 4), 55 per cent ethanol (Fig. 5), and 60 per cent ethanol (Fig. 6).

solvents (Figs. 5 and 6) there are suggestions of minor "protein" impurities which are devoid of biological activity.

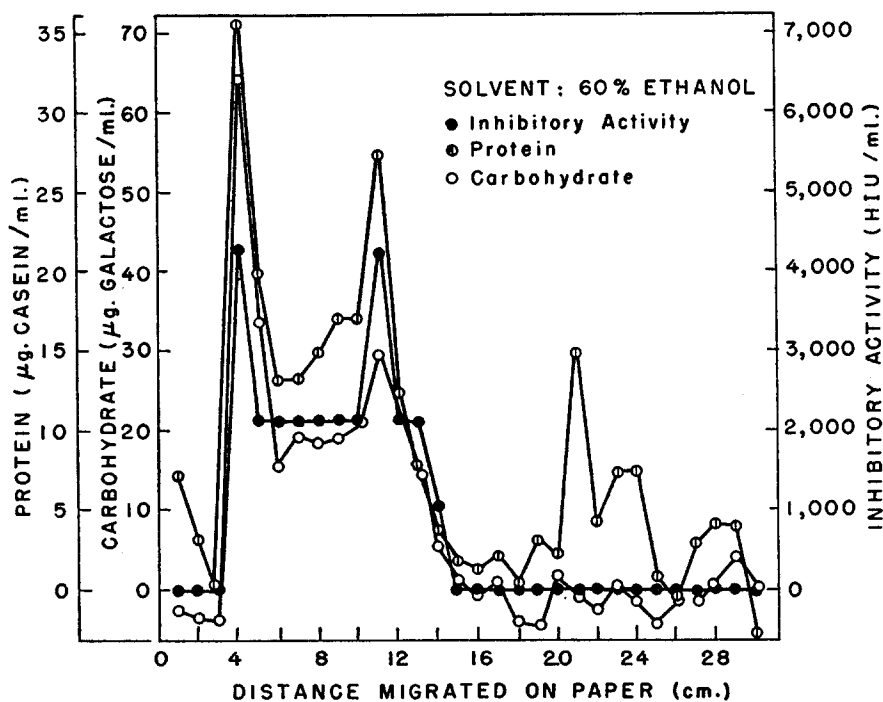


FIG. 6

DISCUSSION

At present the data are too meager to permit any speculation as to the relationship of the chemical nature of the inhibitor to its mode of action. What is perhaps of interest is that this inhibitor is similar in the gross to other biologically active polysaccharide-protein complexes such as the urinary (19) and egg white (20) hemagglutination-inhibitors, a bacteriophage inhibitor derived from Phase II *Shigella sonnei* (21), and the blood group substances (5, 18, 22). That the inhibitor is not a murine blood group substance is shown by the observation that the blood of mice is devoid of inhibitory activity.

SUMMARY

A mucopolysaccharide inhibitor of Theiler's GDVII virus has been purified from intestinal tissue of adult mice. The purified material has been shown to consist chiefly of carbohydrate. Following acid hydrolysis of the inhibitor, several amino acids were found to be present. The carbohydrate components shown to be present thus far are galactose, hexosamine, *N*-acetylhexosamine,

methylpentose, hexuronic acid; an indication has been obtained that sedoheptulose is also present. The available evidence strongly suggests that this mucopolysaccharide, and not some other substance present in minute amounts, is the inhibitor of the GDVII virus.

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