Properties of a Serologically Active Substance from *Leptospira icterohemorrhagiae* (Wijnberg). (20209)

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Information concerning the chemical constitution of leptospires is sparse and contradictory. Failure to find discrete basophilic material within the cells(1) and the low index of refraction characteristic of the organisms led certain workers(2,3) to conclude that the leptospires do not contain nucleoproteins or nucleic acids. This is not in accord with the modern concept(4) of nucleoproteins as necessary participants in the growth and multiplication of cells.

Investigation of the chemical constitution of *Leptospira icterohemorrhagiae* (Wijnberg) in this laboratory indicates that nucleic acids are present in the organisms in appreciable amounts, and that the nucleotide material may be extracted in mixture with a serlogically specific antigen.

Experimental. Leptospira icterohemorrhagiae (Wijnberg) was propagated for 10 to 11 days at 30°C in Stuart's medium(5) enriched with 7.5% normal rabbit serum containing a small amount of hemoglobin, Seitz filtered and incubated at 56°C for 40 minutes prior to use. 200 ml aliquots of medium were seeded with a 20 ml inoculum of a 7-day culture. The leptospires were havested by centrifugation at 20,000 to 80,000 x g, washed twice and resuspended in isotonic phosphate buffered saline solution (pH 7) to 2% of the original culture volume. The average yield of organisms was 64 mg (dry weight) per liter of culture liquid. The resuspended organisms were disintegrated by the addition of an equal volume of a solution containing sodium chloride (0.1 M), sodium citrate (0.2 M) and sodium desoxycholate (0.4%)(6). After standing at room temperature for two hours, the suspension was held at 2-4°C for 18 to 24 hours. After complete disruption of the cells by this process, the suspension was shaken with one-half volume of a mixture of four parts chloroform and one part normal amyl alcohol(7). The biphasic mixture was allowed to stand for several days at 2-4°C, and then separated by low speed centrifugation. The slightly opalescent aqueous phase was carefully separated. The chloroform phase was re-extracted with a small portion of the disrupting fluid and the aqueous extracts combined. The preparation at this stage was designated Fraction 1 and after further purification by three more consecutive extractions with the chloroform mixture was labelled Fraction 1-A. The preparations were dialyzed in Visking cellophane bags against 0.15 M NaCl solution or phosphate buffered saline (pH 7.3), for several days and were reduced in volume by pervaporation. The final volume after dialysis was 3 to 7% of the original volume of the culture. To 25 ml of a preparation of Fraction 1 containing 0.83 mg desoxypentosenucleic acid and 0.55 mg pentose, as d(-) ribose, was added $MgCl_2$ to a concentration of 0.024 M, and 0.5 and 0.2 mg crystalline ribo-and desoxyribonuclease,* respectively. The reaction mixture was incubated at 37°C for 4 hours. The product was transferred to a Visking cellophane bag and then dialyzed in the cold against frequent changes of 0.15 M NaCl solution. The final product contained 0.50 mg pentose. The products were tested as complement-fixing antigens against known hyperimmune rabbit sera by a procedure described by Kolmer, *et al.*,(8), employing one-half volumes. *Total solids* were determined by drying aliquots to constant weight at 95-105°C. The dry weight of the cells and extracted material was estimated as the difference between the weights of the total solids in the preparations and in aliquots of the corresponding diluents. *Total nitrogen* determinations were done by a modified micro-Kjeldahl procedure using a K_2SO_4 –$CuSO_4$ digestion catalyst. *Total pentose* was determined spectrophotometrically with the orcinol re-

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TABLE I. Yield and Composition of Fraction 1 of *Leptospira icterohemorrhagiae* (Wijnberg).*

Preparation	Starting leptospirae		Fraction 1				
	Dry wt of cells	N.	Yield solids	N.	P.	Pentose† (as d-ribose)	DNA‡
	mg						
7	48.6	5.0	23.4	2.2		1.5	1.2
9	123.0	8.0	34.1	2.1		2.2	1.4
10	115.5	6.4	73.1	2.1		2.8	2.2
13	138.6	10.2	54.7	3.3		4.5	4.2
14	192.7	7.5	38.3	3.2		4.3	2.0
15	272.0	20.4	97.4	6.1		8.1	7.8
16	554.2	36.5	120.0	8.4	2.1	10.0	8.2
Total	1444.6	94.0	441.0	27.4		33.4	27.0
% of dry wt		6.51	30.52	1.90		2.31	1.87
% of Fraction 1				6.23		7.58	6.15

* The non-dialyzable leptospiral substance is negative to biuret and ninhydrin tests but gives positive Molisch reaction.

† The colored reaction product of Orcinol-HCl-$FeCl_3$ reagent with Fraction 1 measured spectrophotometrically.

‡ Desoxypentosenucleic acid estimated by a modified procedure of Dische's diphenylamine reaction.

agent of Kerr and Seraidarian(9), using d(-) ribose as the reference standard. Desoxypentosenucleic acid (DNA) was estimated by an adaptation of the Dische diphenylamine reaction(10). *Phosphorus* determinations were made by an adaptation of the method of Gomori(11). The Beckman *DU Spectrophotometer* was used to obtain ultraviolet absorption spectra of the preparations.

Results. Seven preparations of *L. icterohemorrhagiae* (Wijnberg) yielded 1.4446 g dry weight of cells. The total nitrogen content of the leptospires was 94.0 mg or 6.5% (by weight). Fraction 1 contained about 30% of the total solids of the organisms and included an appreciable quantity of a non-dialyzable polynucleotide moiety. The chemical constitution of Fraction 1 is presented in Table I. The non-dialyzable substance containing the active principle from *L. icterohemorrhagiae* shows pronounced serological reactivity with homologous hyperimmune rabbit serum. Cross-reactions of a reduced order are noted in tests with antiserum for *Leptospira canicola*. Serological cross-reactivity with *Leptospira bataviae* antiserum is negligible. Representative data of two preparations were obtained by semi-quantitative complement-fixation titrations and are summarized in Table II. Results of a typical titration are shown in Table III. In some of the tests conducted a well defined zone of inhibition was noted in the region of antigen insufficiency.

TABLE II. Complement-Fixation End-Points of Fraction 1 of *Leptospira icterohemorrhagiae* (Wijnberg).

Antigen diluted 1:	Hyperimmune rabbit sera employed*		
	L. icterohemorrhagiae	*L. canicola*	*L. bataviae*
Preparation 14	Complement-fixation end-points		
1	>650	200	20
2.5	>650	80	10
5	>650	40	1
10	>650	40	1
20	500	10	1
40	1†	10	0
80	0‡	0	0
Preparation 16			
1 §	960	960	960
2.5§	960	480	40
5	960	240	30
10	960	120	15
20	320	30	1
40	160	1	1
80	15	1	0

* Figures represent reciprocals of highest dilutions of antisera reacting with corresponding amounts of the antigen.

† 2+ to 4+ complement-fixation reaction with undiluted antiserum, but complete or nearly complete hemolysis in the next serum dilution tested (1:10).

‡ Complete or nearly complete hemolysis on testing with undiluted antiserum.

§ Slightly anti-complementary at concentrations of antigen indicated.

Enzymatic hydrolysis of Fraction 1 with crystalline ribo-and desoxyribonuclease alters the ultraviolet absorption spectrum, but does

TABLE III. Titration of Fraction 1 of *L. icterohemorrhagiae* with Homologous Hyperimmune Serum.*

Fraction 1 diluted 1:	Homologous hyperimmune serum diluted 1: 1	10	20	40	80	160	320	640
1	4+	4+	4+	4+	4+	4+	2+	—
2.5	4+	4+	4+	4+	4+	4+	4+	—
5	4+	4+	4+	4+	4+	4+	4+†	—
10	3+	3+	4+	4+	4+	4+	4+	—
20	2+	4+	4+	4+	4+	3+	—	—
40	—	2+	2+	3+	±	—	—	—
80	—	—	—	—	—	—	—	—

* According to the method of Kolmer, *et al.*(8).
† Optimal reactivity at a concentration of antigen corresponding to 0.0026 mg of pentose, a dilution of 1 to 380000.

not entirely degrade the polynucleotides. The immunologically-reactive component retains its total activity and remains non-dialyzable. The active principle resists the proteolytic action of trypsin. It is resistant to the action of hemicellulase. After heating the product at 100°C for one hour, there is no appreciable reduction or alteration in immunological activity. Fraction 1 affords negative biuret and ninhydrin tests, but a positive Molisch reaction.

Ultraviolet absorption spectra of Fractions 1 and 1-A (Figure 1) show absorption maxima in the region of 258 millimicrons which is a characteristic property of nucleoproteins and nucleic acids. The absorption spectrum of Fraction 1 resulting from hydrolysis with a mixture of crystalline ribo- and desoxyribonuclease is shown in Fig. 2.

Discussion. The rationale for this study is based on the strong evidence of the association of nucleic acids with dynamic aspects of bio-synthesis of type-specific polysaccharide antigens and properties of virulence of certain microorganisms(12). The procedure applied to the viable cells of *L. icterohemorrhagiae* was selected for use because it involves a type of cellular disintegration which apparently causes negligible damage, if any, to highly polymerized, viscous forms of desoxypentosenucleic acid. The method possesses a distinct advantage. It avoids the dangers of degrading native cellular constituents of the leptospires as might be expected from heating or hydrolysis by strong alkaline and acid reagents. Every effort has been made not to damage the thermostable, serologically active antigen(s) inherent in the living leptospires. Fraction 1 contains, in addition to the active principle, a soluble and non-dialyzable polynucleotide moiety. Whether the immunologically-reactive sub-

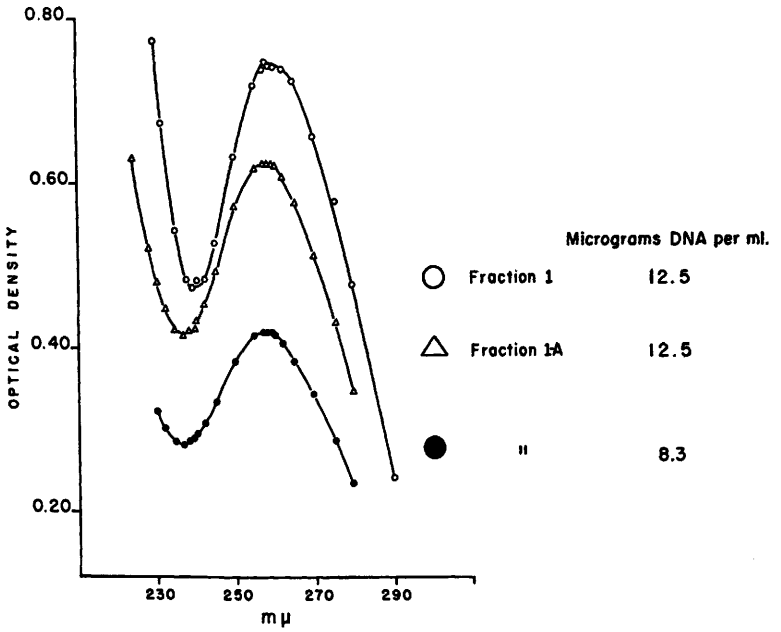

FIG. 1. Ultraviolet absorption spectrum of polynucleotide from *Leptospira icterohemorrhagiae* (Wijnberg).

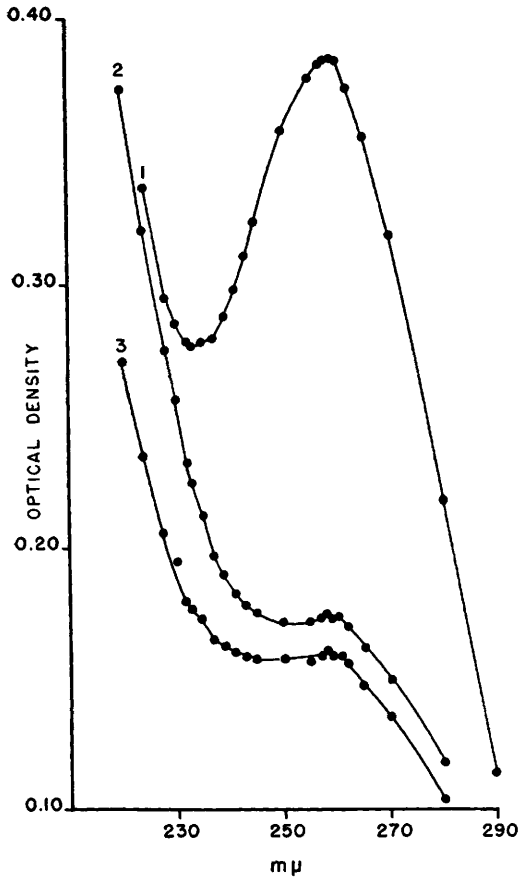

FIG. 2. Ultraviolet absorption spectrum of Fraction 1 of *Leptospira icterohemorrhagiae* before and after hydrolysis with a mixture of crystalline ribo- and desoxyribonuclease. Curve 1. Untreated material at a concentration of 11.1 μg of DNA/ml. Curve 2. Same material after 4 hr hydrolysis with the mixture of the enzymes. Curve 3. The second curve minus the optical density of the enzyme blank. E_o is based on 0.15 M NaCl solution. The complement-fixation antigen was unaffected and reacted at a concentration as low as 0.002 mg pentose/ml, a dilution of 1 to 500000.

stance and polynucleotide exists as a mixture or a chemically-bound complex has not been clearly established. Since crystalline nucleases fail to inactivate the immunological principle, it would appear likely that the polynucleotide is not the determinant of activity of Fraction 1 of *L. icterohemorrhagiae*. The active principle of Fraction 1 is resistant to digestion with trypsin. The latter fact, supported by the failure to react in the biuret test and in a sensitive test like the ninhydrin reaction, virtually indicates that the active substance is not protein.

Reaction products of orcinol and Dische diphenylamine reagents with Fraction 1 indicate the co-existence of pentoses and desoxypentose-nucleic acid.† Enzymatic treatment of the polynucleotide substance singly or with a mixture of crystalline ribo- and desoxyribonuclease is accompanied by a marked decrease in absorption at 258 mμ, thus qualitatively confirming the presence of both types of polynucleotide. Since the cells were grown on a medium devoid of nucleotides and were thoroughly washed prior to extraction, it may be concluded on the basis of the evidence presented that the organism of *L. icterohemorrhagiae* contains substantial amounts of polynucleotide material.

Pentose may be a constituent of the determinant of serological reactivity. Concentrations of 5.5 to 11.1 μg of pentose, as (d-) ribose, per ml of antigen (of which 0.25 ml is employed in the complement-fixation test) have repeatedly given maximal activity with homologous hyperimmune serum.

The presence of immunologically-reactive nucleoproteins and serologically-active pentose-associated polysaccharides is not a rarity among bacteria(13-17). The leptospires do not appear to differ greatly from other bacteria in this respect, a fact which may have some bearing on their disputed classification(3). The chemical complexity of the leptospires was noted at an early date by Noguchi(18). The latter author clearly established the existence of distinct serological types in this group of spirochaetes.

The chemical nature and properties of the determinant of immunological reactivity associated with Fraction 1 of *L. icterohemorrhagiae* and similar fractions from other serotypes of leptospires are now under study in this laboratory.

Summary. Suspensions of viable cells of *Leptospira icterohemorrhagiae* (Wijnberg) were disrupted with an equal volume of a solution containing 0.1 M NaCl, 0.2 M sodium citrate, and 0.4% sodium desoxycholate. An immunologically- reactive, non-dialyzable sub-

† A small amount of thymine was obtained out of impure starting material and was identified by chromatographic and spectrophotometric analyses.

stance (Fraction 1) was prepared by a procedure which avoided heating and use of alkalis and acids. The active principle was isolated in mixture with a non-dialyzable polynucleotide moiety. The organism of *L. icterohemorrhagiae* contained an appreciable amount of the polynucleotide substance and was composed of pentose—as well as desoxypentosenucleic acids. Crystalline ribo- and desoxyribonuclease altered the absorption spectrum of the nucleotide, but failed to inactivate the antigen. On the basis of the latter evidence it appeared likely that the polynucleotide was not the determinant of immunological reactivity. The active principle resisted the action of trypsin, hemicellulase and was not destroyed by heating for 1 hour at 100°C.

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Effect of Pantothenate Deficiency on Synthesis of Adrenal Cholesterol Following Stress.* (20210)

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The occurrence of pathological changes in the adrenal cortex of young rats maintained on diets deficient in pantothenic acid is well established(1-3). The functional capacity of the adrenal cortex is also impaired in this deficiency as indicated by the absence of a lymphopenia following swimming or ACTH (4,5), the absence of an eosinopenia following epinephrine or ACTH(5), and a decrease in glycogen deposition in the liver during fasting or anoxia(6,7). It has been inferred that this functional impairment may be related, at least in part, to low levels of adrenal steroids in the

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