241. *The Constitution of a Lipoid-bound Polysaccharide from* M. tuberculosis (*Human Strain*).

By Sir Norman Haworth, P. W. Kent, and M. Stacey.

The lipoid-bound specific polysaccharide fraction ($[\alpha]_D^{18°}$ + 25°) of *M. tuberculosis* was obtained from lipoid complexes extracted from the heat-killed organisms by organic bases.

The polysaccharide was converted into the methyl ether which on acid hydrolysis furnished 2 : 3 : 5-trimethyl methyl-D-arabofuranoside (12·8%), 3 : 5-dimethyl methyl-D-arabofuranoside (30·9%), 2 : 3 : 6-trimethyl methyl-D-galactopyranoside (33·8%), 3 : 4-dimethyl methyl-D-mannopyranoside (14·4%), and a dimethyl methyl-D-glucosaminide (8%).

The polysaccharide is shown to have a highly branched chain structure containing units of D-galactopyranose, D-mannopyranose, D-glucosamine, and D-arabofuranose, some of the last being present as end groups.

In an investigation of complex substances of possible antigenic activity derived from *M. tuberculosis* (human strain), it was found that fractions could be separated from heat-killed cells by the action of basic organic substances (*e.g.*, urea, β-hydroxypropionamidine, etc.). Some of the fractions were highly serologically active when tested by the collodion particle agglutination test (Cannon and Marshall, *J. Immunol.*, 1940, **38**, 365; Riodoran, *Proc. Soc. Exp. Biol. Med.*, 1942, **49**, 622).

It was found that the most active fractions contained lipoids, deoxyribonucleic acid, and a serologically specific polysaccharide ($[\alpha]_D^{18°}$ + 25°), which had properties identical with the lipoid-bound polysaccharide isolated previously (Haworth, Kent, and Stacey, preceding paper) by alkaline extraction of *M. tuberculosis* cells.

The lipoid-bound polysaccharide obtained either by fractionation of the mixed polysaccharide of defatted cells or by alkaline treatment of the antigenic complex (see experimental section) was methylated with sodium hydroxide and methyl sulphate. After six treatments with the reagents the crude methylated derivative had a constant methoxyl content of *ca.* 42% and was in the form of a brown gum which was fractionated by precipitation from organic solvents.

This methylated derivative (20·5 g.) was hydrolysed by being refluxed with methanolic hydrogen chloride ($[\alpha]_D^{17°}$ + 31·5° ⟶ + 17°, 9 hours). The resulting mixture of glycosides was fractionated by distillation in a high vacuum. Five substances were identified :

(*a*) 2 : 3 : 5-Trimethyl methyl-D-arabinoside, identified by oxidation with bromine to the corresponding lactone and thence to 2 : 3 : 5-trimethyl D-arabonamide.

(*b*) A fraction (OMe, 48·5%) which underwent further methylation when treated with silver oxide and methyl iodide. Oxidation of the completely methylated product (OMe, 60%) afforded a lactone which was readily converted into 2 : 3 : 5-trimethyl D-arabonamide.

The initial fraction (*b*) was hydrolysed with N/20-sulphuric acid ($[\alpha]_D^{17°}$ − 6° ⟶ − 40°, 6 hours) which suggested the possible existence of a furanose structure. Treatment of a specimen of the free sugar derivative with alcoholic aniline yielded a crystalline anilide identical with the dimethyl pentose anilide from the methylated somatic polysaccharide (see previous paper).

The free sugar was oxidised with bromine, and the lactone so formed was converted into 3 : 5-dimethyl D-arabonamide.

(*c*) A fraction, having properties corresponding to those of a trimethyl methylhexoside, was methylated further, and hydrolysed to the free sugar which was then converted into 2 : 3 : 4 : 6-tetramethyl D-galactose anilide.

The original fraction (*c*) was hydrolysed directly to the free sugar which was oxidised to the acid. On being distilled in a high vacuum a crystalline 2 : 3 : 6-trimethyl D-galactonolactone was obtained (cf. Haworth, Raistrick, and Stacey, *Biochem. J.*, 1935, **29**, 2668).

(*d*) A sample of a fraction having OMe, 43·6% was methylated, and converted into 2 : 3 : 4 : 6-tetramethyl D-mannose anilide.

A further sample of this fraction (*d*) was hydrolysed. The free sugar crystallised on standing and was identified as 3 : 4-dimethyl D-mannopyranose.

(*e*) The amino-sugar constituent of the hydrolysed methylated polysaccharide was separated in the following manner. The acidic methanolic hydrolysate was diluted with water, the methanol removed by evaporation, and thereafter the mixed glycosidic constituents were obtained from the solution by continuous extraction with chloroform. The partially methylated amino-sugar hydrochloride remained in the aqueous liquid. The hydrochloride was isolated as a syrup. After acetylation of the amino-group and methylation of free hydroxyl groups, *N*-acetyl 3 : 4 : 6-trimethyl α-methyl-D-glucosaminide was obtained.

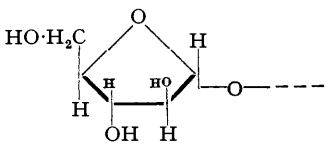
Examination of all the syrups obtained by distillation afforded only further amounts of these five components.

Discussion.

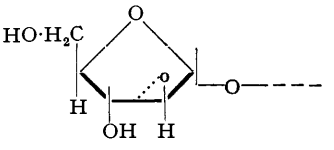
A quantitative assay of these constituents was made by reference to the refractive indices and methoxyl and nitrogen contents of the various glycosidic fractions. The following results were obtained: 2 : 3 : 5-trimethyl methyl-D-arabinoside (12·8%); 3 : 5-dimethyl methyl-D-arabinoside (30·9%); 2 : 3 : 6-trimethyl methyl-D-galactoside (33·8%); 3 : 4-dimethyl methyl-D-mannoside (14·4%); dimethyl methyl-D-glucosaminide (8%). This corresponded to a molecular ratio of 2 : 5 : 5 : 2 : 1 respectively.

The identification of 2 : 3 : 5-trimethyl methyl-D-arabinoside as a constituent of the methylated polysaccharide indicated that D-arabofuranose is combined glycosidically and is therefore an end group of the polysaccharide linked as in (I).

Since 3 : 5-dimethyl methyl-D-arabinoside was also present, it is clear that further D-arabofuranose residues are combined in the polysaccharide chain and are linked through the 1 and 2 positions as in (II).



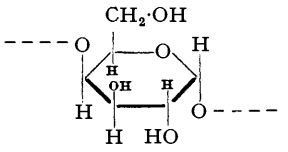
(I.)



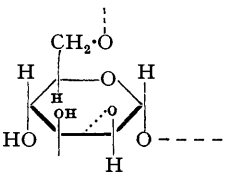
(II.)

The occurrence of 2 : 3 : 6-trimethyl methyl-D-galactoside establishes that D-galactose is present in the polysaccharide chain and is probably linked through positions 1 and 4 as in (III).

D-Mannopyranose is linked through the 1, 2, and 6 positions in the polysaccharide (IV), and reflects the degree of branching.



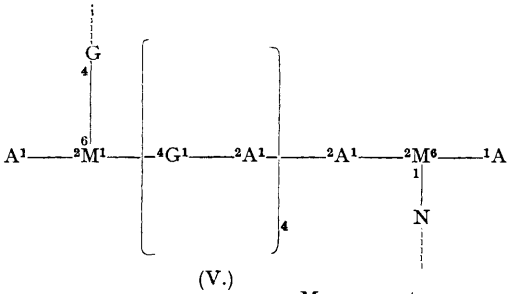
(III.)



(IV.)

The precise mode of linkage of the glucosamine residue which forms part of the chain has not yet been determined.

It is possible, in view of these results, to depict several possible configurations for this polysaccharide, one type of which is given in (V).



(V.)

A represents D-arabofuranose.
G represents D-galactopyranose.
M represents D-mannopyranose.
N represents D-glucosamine.

In this representation the linkages engaged glycosidically at C_1 are provisionally shown as being in the α-form in view of the change of rotation on hydrolysis of both the polysaccharide ($[\alpha]_D^{18°}$ + 28° ⟶ − 16°) and its methylated derivative ($[\alpha]_D^{18°}$ + 30° ⟶ − 15°) which are in the negative sense.

Work on determination of the molecular weight of this polysaccharide by osmotic pressure and oxidation methods is proceeding. The periodic oxidation method gives results which are less satisfactory than those for the somatic polysaccharide.

Experimental.

Isolation of the Complex.—Moist heat-killed cells were stirred with an equal weight of urea and warmed at 37° for 100 hours. The residual fluid was diluted with water (½ vol.) and centrifuged (2,500 r.p.m.) until no further solid material separated. To the supernatant liquid acidified (litmus) with dilute acetic acid, alcohol (5 vols.) was added, and the precipitated solid at once removed. This moist product was shaken with an equal volume of a 2% solution of sodium acetate and centrifuged. The residual solid was a complex consisting of free lipoid (soluble in organic solvents), a specific polysaccharide, $[\alpha]_D^{20°}$ + 28°, bound lipoids, and deoxyribonucleic acid (the last could be separated by extraction with dilute sodium hydroxide). The complex, as will be described elsewhere, gave, when combined with collodion, a strong agglutination reaction with sera from tuberculous patients.

Composition of the Serologically Active Urea Extract.—(i) *Determination of ether-soluble lipoid content.* The serologically active complex (4·4 g.) was extracted continuously for 30 hours with ether (1 l.) in a Soxhlet apparatus. Evaporation of the ethereal extract yielded a residue of lipoid material (0·628 g., 27%).

The initial fraction was acid-fast when stained by the carbolfuschin method. After the ether treatment, the defatted residue was non-acid fast though this property was retained by the extracted lipoids.

(ii) *Alkaline hydrolysis.* The ether-extracted fraction (2·5 g., 9·4%N) was heated at 100° for 30 hours with sodium hydroxide (40 c.c. of N). The unsaponified residue (1·1 g.) was removed and the supernatant liquid made acid (litmus). The precipitate, which consisted of peptide material, was collected, and alcohol (4 vols.) was added to the acidified mother liquor. The resulting precipitate was separated, dissolved in a small volume of water, dialysed through cellophane, and finally reprecipitated. The precipitate, which contained 4·7% of nitrogen, gave strong Molisch and Dische tests, and appeared to be composed of polysaccharide substances and deoxyribonucleic acid. A further fractionation was therefore carried out in order to separate the constituents of the mixture (see iii, below).

The alcoholic mother liquors, containing those fatty residues liberated during the saponification, were evaporated to dryness. A small amount (0·13 g.) of bound lipoid residues was obtained; the properties of this will be described elsewhere.

(iii) *Fractionation of the polysaccharide component.* The crude polysaccharide-containing precipitate isolated above was dissolved in water (50 c.c.), and alcohol was added dropwise to this aqueous solution, with stirring until a precipitate *F*1 was observed. This was collected and more alcohol was added, in the same manner as before, to the supernatant liquor, furnishing a precipitate *F*2. The procedure was continued until no further fractions were precipitated (Table I).

Table I.

No.	Wt. (g.).	Ash, %.	$[\alpha]_D^{18°}$.	Biuret.	Molisch.	Dische.	Bial.
*F*1	0·177	47·7	± 0°	—	++	+++	++
*F*2	0·065	26·0	+30	—	++	±	+
*F*3	0·119	19·9	+28	—	++	—	+
*F*4	0·06	1·8	+27	—	+	—	+
*F*5	0·03	2·1	+29	—	+	—	+
*F*6	0·18	35·0	+26	—	+	—	+

The crude polysaccharide mixture and the polysaccharide fraction *F*3, reacted in the precipitin test with sera from tuberculous patients in the following dilutions:

Crude polysaccharide	1 : 1,000,000
Fraction *F*3	1 : 2,000,000

Methylation of the Lipoid-bound Polysaccharide ($[\alpha]_D^{18°}$ + 25° in water).—The fractionated material (8 g.; $[\alpha]_D^{18°}$ + 25°) was dissolved in water (25 c.c.) and treated with sodium hydroxide (500 c.c. of 30%) and methyl sulphate (185 c.c.) in aliquot tenth portions every 10 minutes at 35° with vigorous stirring. Sufficient sodium hydroxide was first added to maintain alkaline conditions throughout. The mixture was stirred for 2 hours after all the reagents had been added, then cooled in ice and acidified (Congo-red) with dilute sulphuric acid. A quantity of methylated material separated at this stage. The aqueous solution was neutralised, dialysed (parchment) for 48 hours, then concentrated to a small volume (30 c.c.). This liquor was mixed with the methylated derivative which separated on acidification and treated with methyl sulphate and sodium hydroxide in the manner described.

After 3 such treatments most of the methylated derivative separated on acidification, and any remaining material was extracted with chloroform. In this way, dialysis of the aqueous liquors was not necessary. The product was dried in a vacuum and dissolved in dry pyridine (70 c.c.) and acetic anhydride (10 c.c.). After 48 hours at room temperature, the solvents were removed under reduced pressure (at room temperature) and the product again methylated. The methoxyl content was constant

after 6 methylations (yield 6 g.). By repetition of this procedure, 29 g. of methylated derivative were isolated.

Fractionation of the Methylated Polysaccharide.—The derivative (6·5 g.) was fractionated by graded precipitation from a chloroform solution (40 c.c.) with light petroleum. Five fractions were thus obtained and a further fraction was separated by evaporation of the mother liquors (Table II).

TABLE II.

No.	Petroleum added (c.c.).	Wt. (g.).	$[\alpha]_D^{20°}$.	OMe, %.	
1	—	0·9	—	—	inorganic residue
2	200	0·6	+ 9°	25·6	
3	98	2·9	+29	42·8	mainly methylated polysaccaride
4	102	0·5	+32	42·1	
5	135	0·9	+30	42·3	
6	evaporation	0·6	+25	31·0	

In this way, 21 g. of essentially homogeneous methylated polysaccharide were isolated, having N, 0·4; OMe, 42·3%; P, nil; and $[\alpha]_D^{19°}$ + 30°. Attempts to increase the methoxyl content of the substance by treatment with silver oxide and methyl iodide were unsuccessful, and it was therefore concluded that the product was already fully methylated.

Hydrolysis of the Methylated Polysaccharide.—A specimen (0·22 g.) was refluxed with methanolic hydrogen chloride (25 c.c.; 1%). The rate of hydrolysis was followed polarimetrically and was constant after 9 hours ($[\alpha]_D^{19°}$ + 31·5° ⟶ + 17°). When a further sample (0·10 g.) was hydrolysed with 5% methanolic hydrogen chloride, the reaction was completed in 1½ hours ($[\alpha]_D^{20°}$ + 34·0° ⟶ + 15·1°). There was no further change in optical rotation when the concentration of the acid was increased to 8%. The acid was neutralised with silver carbonate, and, after filtration, evaporation of the alcoholic solution gave a mixture of glycosides. From 20·5 g. of methylated polysaccharide, 19·5 g. of glycosidic hydrolysis products were obtained.

Fractional distillation of the products of hydrolysis. The mixture of glycosides (16·1 g.) was fractionally distilled from a Widmer flask in a high vacuum. Seven fractions were thus separated (Table III).

The still residue was rehydrolysed with methanolic hydrogen chloride (50 c.c.; 6%) for 4 hours, and the hydrolysis product again distilled, thus furnishing 6 further fractions (Table IV).

TABLE III.

No.	Wt. (g.).	Vap. temp.	Bath temp.	$n_D^{18°}$.	OMe, %.
1	1·31	66°	97—108°	1·4381	58·4
2	0·70	66— 84	109—129	1·4490	53·0
3	3·81	84— 90	129—132	1·4520	48·5
4	2·10	90— 95	132—154	1·4531	52·0
5	2·00	96—102	154—166	1·4520	50·1
6	1·13	102—117	175—189	1·4600	46·4
7	0·71	117—121	189—210	1·4705	43·6

TABLE IV.

Rehydrolysis of still residue.

No.	Wt. (g.).	Vap. temp.	Bath temp.	$n_D^{18°}$.	OMe, %.
8	0·04	55°	110°	1·4419	60·3
9	0·65	87— 89	122—125	1·4468	55·4
10	0·84	106—115	143—155	1·4533	53·6
11	0·43	104—106	155—163	1·4589	45·4
12	0·38	110—111	196—200	1·4660	45·6
13	0·85	120—130	220—240	1·4829	40·7

Still residue 0·9 g. Pressure 0·04 mm.

Identification of 2 : 3 : 5-*trimethyl methyl*-D-*arabinoside.* A sample of the fraction 1 (0·5 g.) was hydrolysed with N/20-sulphuric acid (25 c.c.) at 100° ($[\alpha]_D^{17°}$ + 63° ⟶ + 26°; 6 hours). The acid was neutralised (phenolphthalein) with sodium hydroxide, the solution evaporated to dryness, and the free sugar (0·45 g.; $n_D^{17°}$ 1·4470; OMe, 49·8%) extracted with ether. This sugar was oxidised by treatment with bromine (1 c.c.) at room temperature for 3 days. After aeration to remove unchanged bromine, the solution was neutralised with silver carbonate, filtered, and treated with hydrogen sulphide. After filtration, the liquid, which was non-reducing to Fehling's solution, was evaporated to dryness. The free acid so obtained was extracted with chloroform and lactonised by being heated at 100° for 3 hours in a vacuum.

The syrupy lactone was treated with dry methanolic ammonia (10 c.c.) for 12 hours at room temperature. On evaporation to dryness a syrup was obtained which crystallised rapidly on nucleation with 2 : 3 : 5-trimethyl D-arabonamide; m. p. 134—135° alone or in admixture with an authentic specimen (Found: OMe, 43·2%; $[\alpha]_D^{18°}$ − 25° in alcohol).

Identification of 3 : 5-*dimethyl methyl*-D-*arabinoside.* A specimen of fraction 3 (0·65 g.) was methylated by 3 treatments with methyl iodide and silver oxide. The fully methylated product (0·4 g.; b. p. 93—98°/0·2 mm.; $n_D^{19°}$ 1·4381; OMe, 60·8%) was hydrolysed with N/10-hydrobromic acid

($[\alpha]_D^{17°}$ − 3·5° ⟶ − 10°; 4 hours) and the free sugar oxidised with bromine. The lactone (0·2 g.; n_D 1·4590) was converted into the amide by treatment for 12 hours with saturated methanolic ammonia (5 c.c.). The product was crystallised from acetone; m. p. 135° alone or in admixture with 2 : 3 : 5-trimethyl D-arabonamide (OMe, 45·1%; $[\alpha]_D^{16°}$ − 13° in water).

A further specimen (1·1 g.) of the fraction 3 was hydrolysed by being heated with N/20-sulphuric acid (20 c.c.) at 100° for 9 hours ($[\alpha]_D^{18°}$ − 6·1° ⟶ − 40·0°). Some of the free sugar (0·08 g.; $n_D^{16°}$ 1·4712; OMe, 35·3%) was refluxed with alcoholic aniline (0·04 g. in 2 c.c.) for 6 hours. The anilide was crystallised from acetone–light petroleum; m. p. 118° (Found: OMe, 24·0. Calc. for a dimethyl pentose anilide: OMe, 23·1%).

The free sugar (0·6 g.) was oxidised with bromine (1·5 c.c.) for 4 days at room temperature. The acid, isolated in the usual way, was distilled [0·3 g.; b. p. 176° (bath temp.)/1 mm.; $n_D^{18°}$ 1·4521; OMe, 37·1%] and this product—a lactone—converted into the amide with methanolic ammonia and crystallised from acetone; m. p. 144°; OMe, 33%; $[\alpha]_D^{17°}$ − 10° in water [cf. m. p. 145°; $[\alpha]_D^{17}$ + 10° for 3 : 5-dimethyl L-arabonamide (Cunneen, Ph.D.Thesis, Birmingham University, 1940; White, *J. Amer. Chem. Soc.*, 1946, **68**, 272)].

Identification of 2 : 3 : 6-*trimethyl methyl*-D-*galactoside.* A sample of the mixed fractions 4, 5, 10 (0·5 g.) was methylated with silver oxide and methyl iodide. The product (0·4 g.; $n_D^{18°}$ 1·4420; OMe, 61·2%) was hydrolysed with N-sulphuric acid (10 c.c.) at 100° for 7 hours ($[\alpha]_D^{20°}$ + 0·6° ⟶ − 2·5°). The free sugar (0·2 g.; $n_D^{19°}$ 1·4560; OMe, 50·1%) was refluxed with alcoholic aniline (0·1 g. in 4 c.c.) for 6 hours. The anilide was crystallised from acetone–light petroleum; m. p. 186° alone or in admixture with an authentic specimen of 2 : 3 : 4 : 6-tetramethyl D-galactose anilide.

A further specimen of the mixed fraction (0·6 g.) was hydrolysed by being heated with N-sulphuric acid (20 c.c.) at 100° for 8 hours ($[\alpha]_D^{18°}$ − 4·0° ⟶ − 9·0°). The free sugar (0·3 g.; $n_D^{18°}$ 1·4620) was oxidised with bromine (1 g.) at room temperature for 5 days. On distillation of the acid so formed (b. p. 158°/0·02 mm.) a lactone was obtained which crystallised on being nucleated with 2 : 3 : 6-trimethyl D-galactonolactone. The material was recrystallised from ether–ethanol and had m. p. 96° alone or in admixture with 2 : 3 : 6-trimethyl D-galactonolactone (Found: OMe, 42·7. Calc. for $C_9H_{16}O_6$: OMe, 42·3%).

Identification of 3 : 4-*dimethyl methyl*-D-*mannoside.* A specimen (0·5 g.) of the mixed fractions 6, 11, 12 was methylated by 3 treatments with methyl iodide and silver oxide. The fully methylated product (0·45 g.; $n_D^{16°}$ 1·4460; OMe, 61·4%) was hydrolysed with N-sulphuric acid (10 c.c.) in 5 hours ($[\alpha]_D^{17°}$ + 6° ⟶ ± 0°). The free sugar (0·2 g.; $n_D^{17°}$ 1·4579; OMe, 52·3%) was converted into the anilide by being boiled with aniline (0·18 g.) in dry ethanol (6 c.c.) for 6 hours. Evaporation of the solvents yielded an anilide which was recrystallised from acetone–light petroleum; m. p. 138° alone or in admixture with an authentic specimen of 2 : 3 : 4 : 6-tetramethyl D-mannose anilide.

A specimen (0·5 g.) of the mixed fraction was hydrolysed ($[\alpha]_D$ + 13° ⟶ + 8°; 9 hours) with N-sulphuric acid (20 c.c.). The free sugar which crystallised on standing at 0° from ether–ethanol, had m. p. 108° alone or in admixture with 3 : 4-dimethyl D-mannose (Found: OMe, 29·9. Calc. for $C_8H_{16}O_6$: OMe, 29·8%).

Investigation of the Amino-sugar Constituent.—Methylated lipoid-bound polysaccharide [7 g.; OMe, 42·2%; $[\alpha]_D^{18°}$ + 30° in chloroform (*c*, 0·4)] was hydrolysed by being boiled with methanolic hydrogen chloride (10% : 100 c.c.) for 36 hours. Water (100 c.c.) was added to the hydrolysate, and the methanol removed under reduced pressure. The acid aqueous solution so obtained was extracted continuously with chloroform for 80 hours. The aqueous layer was separated and concentrated in presence of solid sodium hydroxide. A syrup (0·3 g.; OMe, 35·8%) was obtained, which, after removal of a small amount of inorganic impurity, was acetylated by being shaken with acetic anhydride (0·5 g.) and dry silver acetate (0·6 g.) in dry methanol (20 c.c.). The mixture was shaken overnight at room temperature, and after filtration the solution was evaporated to dryness. The resulting syrup (0·15 g.) was methylated 5 times using silver oxide and methyl iodide. A crystalline solid was obtained having m. p. 150° alone or in admixture with *N*-acetyl 3 : 4 : 6-trimethyl α-methyl-D-glucosaminide (Found: OMe, 44·9. Calc. for $C_{12}H_{23}O_6N$: OMe, 44·8%) (cf. Cutler, Haworth, and Peat, *J.*, 1937, 1979).

Assessment of the Methylated Sugar Components of the Methylated Polysaccharide.

Distillate weight 14·47 g. Still residue 0·9 g.

Component.	Wt. (g.).	% (by wt.).	*M.*	Mol. proportion.	% (mol.).	Molecular ratio.
2 : 3 : 5-Trimethyl methyl-D-arabinoside	1·86	12·83	206	9·03	13·5	2·05
3 : 5-Dimethyl methyl-D-arabinoside	4·48	30·95	192	23·33	34·9	5·3
2 : 3 : 6-Trimethyl methyl-D-galactoside	4·89	33·78	236	20·72	31·0	4·7
3 : 4-Dimethyl methyl-D-mannoside	2·08	14·37	222	9·36	14·0	2·1
Dimethyl methylglucosaminide	1·16	8·02	263·5	4·40	6·6	1·0

The serological tests were carried out by our colleague, Dr. E. Nassau.

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