AN ANTIGENIC POLYSACCHARIDE, "POLYSACCHARIDE II" ISOLATED FROM TUBERCULIN

by

FLORENCE B. SEIBERT, M. STACEY, AND P. W. KENT

The Henry Phipps Institute, University of Pennsylvania, Philadelphia, and the Department of Chemistry, University of Birmingham (England)

Considerable information has been gained concerning a polysaccharide found in tuberculin (LAIDLAW AND DUDLEY, 1925; MUELLER, 1926; RENFREW, 1930; STACEY AND KENT, 1948). It was shown (SEIBERT, PEDERSEN, AND TISELIUS, 1938; TENNENT AND WATSON, 1942) to have a molecular weight of about 7000–9000 to consist of units of mannose, galactose, and arabinose (RENFREW, 1930), and to be ineffective in causing a tuberculin skin reaction (MCCARTER AND WATSON, 1942).

Recently we have discovered in tuberculin another polysaccharide with very different properties, and it has been designated "Polysaccharide II" to distinguish it from the former one which has now been called "Polysaccharide I". It is the purpose of this paper to describe some chemical and serological properties of Polysaccharide II.

EXPERIMENTAL

OCCURRENCE

Polysaccharide II was first suspected when we observed in the Tiselius an extra sharp peak with very low electrophoretic mobility on several tuberculin preparations made from a bovine strain (# 523) tubercle bacillus and was, therefore, at first thought to be characteristic of the bovine strain. Since then, however, it has been found in filtrates from three human strains as well as from the B.C.G. strain and, therefore, cannot be considered specific for the bovine type bacillus. Certain tuberculin filtrates have an opalescent appearance which has been shown to be due not to a contaminant but rather to the presence of this polysaccharide, for it was later found that aqueous solutions of the isolated polysaccharide are very opalescent. Moreover, the same strain may yield a tuberculin filtrate containing large amounts of this Polysaccharide II at one time while at other times it is absent. For example, during earlier studies made with the H 37 human strain tubercle bacillus some years ago, the filtrates were clear and none of this polysaccharide was detected, whereas in recent months what is presumably the same strain yields large quantities though it may be significant that during this time the strain has become relatively avirulent. Furthermore, one strain "DT", which is a considerably more virulent human strain, gives a clear filtrate from which only a

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very small amount of the Polysaccharide II can be isolated; an amount so small that it would probably be missed if it were not directly sought by special methods.

ISOLATION

Polysaccharide II has so far been isolated from five different strains, two bovine, # 523 and B.C.G., and from three human strains, H 37, A 33, and DT. Essentially the same method of isolation was used in all cases, which was as follows:

The organisms were grown for 8 to 12 weeks on LONG's synthetic medium and quickly filtered off alive on paper and then passed through a Seitz filter. The filtrates were taken to a cold room maintained at 1–2° C and all further procedures were carried out at this temperature. The sterile filtrates were concentrated by ultrafiltration to about one tenth of their original volume and the filtered solution adjusted to about p_H 4.0 with acetic acid. The resulting precipitates, which consisted mainly of protein C (SEIBERT, 1949), were removed by centrifugation. The supernatants were filtered if not clear, readjusted back to p_H 7.0 with alkali, and sufficient alcohol added to give a concentration of 30%. The resulting precipitates consisted essentially of Polysaccharide II, which formed very opalescent solutions when dissolved in water and gave white powders when dried.

The yields varied considerably with the strain, as mentioned above (see Table I). The bovine # 523, H 37 and A 33 strains yielded significant amounts of this polysaccharide, whereas only small amounts were obtained from the B.C.G. and DT strains.

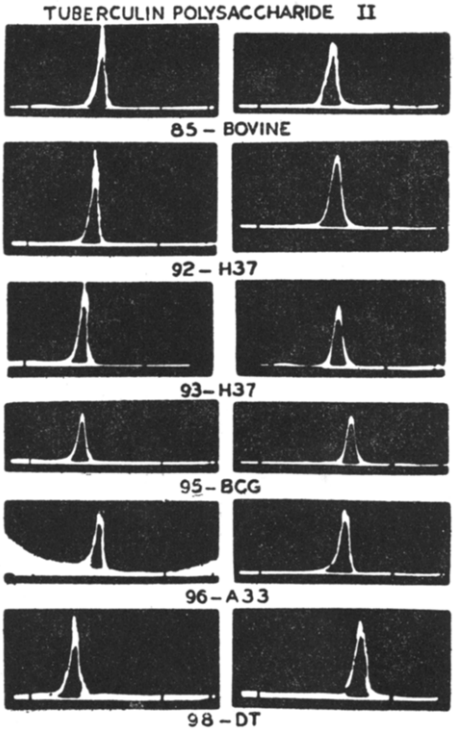

Fig 1

CHEMICAL PROPERTIES

Electrophoresis. When solutions of the pure isolated polysaccharides were studied by the Tiselius electrophoresis technique in phosphate buffer p_H 7.7, $\mu = 0.1$, they were found to have very slow mobilities of —1.0 to $-1.6 \cdot 10^{-5}$ cm^2 volt^{-1}sec^{-1}, and to consist of a single component only, even after four hours of electrophoresis at a potential gradient of 7 to 9 V per cm (see Fig. 1 and Table I). The peaks remained very sharp on both ascending and descending sides and were in great contrast to the low spreading

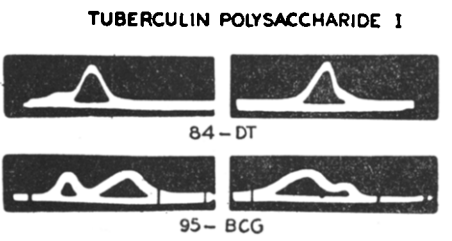

Fig. 2

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TABLE I

ISOLATION AND YIELDS OF POLYSACCHARIDE II

Strain of tubercle bacilli used	LONG's medium used (l)	Age in weeks	Concentrated to ml	Precipitated at p_H	Readjusted to p_H	Treatment of supernatant	Precipitated with alcohol %	Yield in g	Electrophoretic mobility* (10^5 cm^2 volt^{-1} sec^{-1})
85 (Bov.)	40	?	213	3.7	7.0	Pptd with 213 ml in neutral $(NH_4)_2SO_4$ Later treated with charcoal	55	2.46	—1.0
92 (H 37)	11.8	9½	820 (used 790)	4.0	6.5	Concd to 455 ml	20	1.79	—1.4
93 (H 37)	4.5	9	540	4.1	7.0		30	0.86	—1.5
95 (BCG)	26.5	8	1000	4.0	7.0	Concd to 426 ml Pptd with 426 ml neutral $(NH_4)_2SO_4$ Sup. concd	30	0.09	—1.5
96 (A 33)	24.5	12	730	4.7 and 3.8		Ppt 30% alc. redissolved p_H 7.6	30	0.70	—1.6
98 (DT)	28.7	8½	850	4.4	7.8		30	0.124	—1.6

* In phosphate buffer p_H 7.7 = 0.1

curves found for the Polysaccharide I preparations (see Fig. 2), studied with similar concentrations and conditions.

Even in the presence of other components of tuberculin, Polysaccharide II is clearly distinguishable on the electrophoretic diagrams, due to its sharp peak. An attempt was, therefore, made to determine the p_H mobility curve in such a mixture,

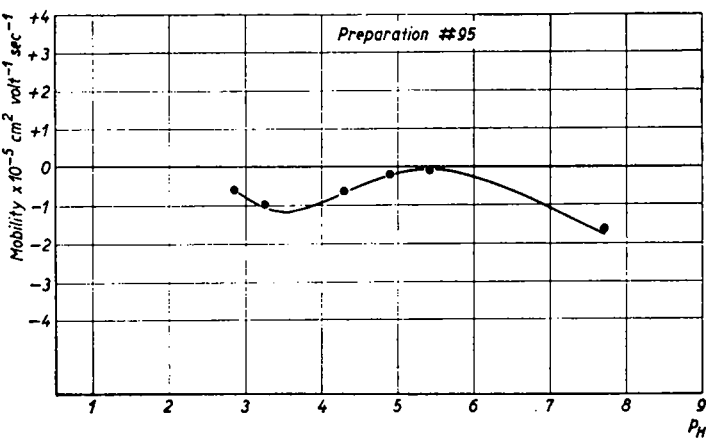

Fig. 3

using citrate, acetate, or phosphate buffers of 0.1 ionic strength. Fig. 3 shows the curve obtained. There was apparently no charge around p_H 5.3, and a slightly acidic charge around p_H 3.5, as well as on the alkaline side of p_H 5.5.

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Sedimentation and diffusion. Two sedimentation measurements were made in the SVEDBERG ultracentrifuge on the polysaccharide (Preparation # 85) by Mrs. ELLEN BEVILACQUA at the University of Wisconsin to whom we are very grateful. The resulting constants were $S_{20} = 2.13$ and 2.09. In both cases the sedimentation curves were symmetrical, indicating considerable homogeneity in the preparation.

Determination by Mrs. BEVILACQUA of the rate of diffusion, using the Lamm diffusion cell, showed a constant of $D_{20} = 1.0$. The diffusion curves measured during the experiment showed considerable skewing at the beginning, but definite symmetry at the end. The constants calculated were as follows.

At 55 h $D_{20} = 2.88$
,, 69 ,, $D_{20} = 1.3$
,, 79 ,, $D_{20} = 1.4$
,, 96 ,, $D_{20} = 1.3$
,, 117 ,, $D_{20} = 1.1$
,, 139 ,, $D_{20} = 1.0$

This preparation was the first one isolated, and on electrophoresis showed a very small extra component with slightly higher mobility than the main polysaccharide component. It perhaps was this small contaminant which caused the skewing of the early diffusion curve. Later preparations were entirely free of this component, as was also a repurified polysaccharide (# 85). See Fig. 1.

The sedimentation and diffusion constants would, therefore, suggest a very elongated chain molecule with high molecular weight, of the order of 100000 and having a high dissymmetry ratio.

Spectrographic absorption. An 0.45% solution of the polysaccharide # 92 was studied in the Beckmann spectrophotometer in the ultraviolet range and Fig. 4 shows there is no definite absorption, except a small amount at wave length λ 2800 Å, indicating possibly the presence of a trace of protein or an amino acid residue. Similar absorption curves have also been obtained with the Polysaccharide I.

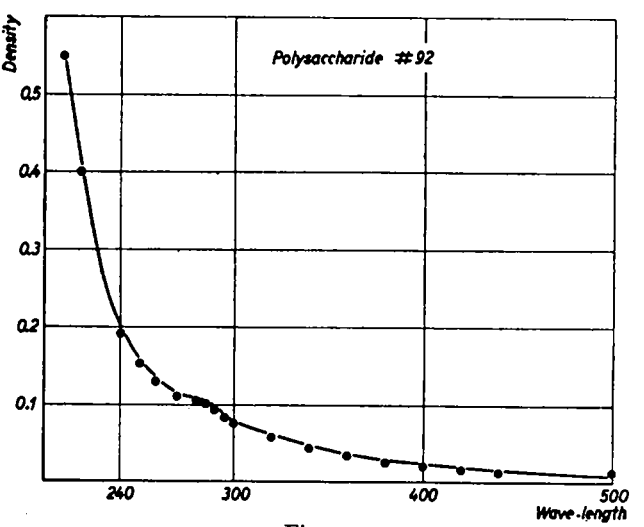

Fig. 4

Chemical analyses and structure. The nitrogen contents of two of the preparations (# 85 and # 92) were determined by the micro-Kjeldahl method and found to be 0.59 and 0.31% respectively. The former result was obtained on the original # 85 preparation, before repurification, and indicates a maximum protein impurity of about 3.3%. For the # 92 preparation it was 1.9%.

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There was however some evidence from the Ehrlich test that an amino sugar was present.

The diphenylamine reaction for deoxyribonucleic acid was negative when applied to about 25 mg of the repurified # 85 preparation, from which observation and from the spectrographic data, it is safe to conclude that there was no nucleic acid present.

When studied by means of the carbazole reaction (SEIBERT AND ATNO, 1946), a deep pink colour, similar to that shown by glucose, was given, in contrast to the yellowish pink given by the Polysaccharide I preparation. The ratio of the density at wave length λ 5400 Å to that at wave length λ 4200 Å was 3.97 for preparation # 85 (original) and 5.30 for preparation # 92. These ratios were close to that given by glucose, 4.89, and very different from the ratio of 1.38 for Polysaccharide I, which has been shown (RENFREW, 1930) to consist of mannose, galactose, and arabinose. It would seem, therefore, that Polysaccharide II might consist mostly of glucose units.

Confirmation of this was found in the following chemical studies on preparation # 92. Qualitatively no pentose or uronic constituents could be found. The optical rotation was $[\alpha]_D^{24}$ + 165° (in water, c = 0.2). Upon hydrolysis with 0.1 N sulphuric acid for 6 hours, there was first a slight increase to $[\alpha]_D^{24}$ + 171° → 186°. The polysaccharide was then readily hydrolysed by being heated at 100° C with N sulphuric acid for 5 hours, $[\alpha]_D^{24}$ + 171° changing to + 58°, a behaviour which resembled that of the polyglucose, dextran.

The mixture of reducing sugars in the hydrolysate was examined chromatographically using the paper-strip technique (PARTRIDGE, 1946). By means of a butanol-ethanol-water mixture as the eluting agent, it was shown that the mixture contained two principal reducing constituents. Comparison of the R_f values of the two constituents indicated that one of them was glucose, and this was further confirmed by including authentic glucose in the paperstrip chromatogram.

The second slower-moving constituent sugar has not yet been identified.

An attempt was made to separate and characterize constituent sugars of the polysaccharide hydrolysate by the formation of crystalline derivatives. The mixture of reducing sugars obtained as described was treated with ethyl mercaptan in strongly acid solution at 0° C (WOLFROM AND KARABINOS, 1945). The mixed mercaptals so formed were acetylated in pyridine and acetic anhydride. This yielded two products, one a crystalline substance and the other a syrup (in small quantity). The crystalline diethyl mercaptal pentacetate had m.p. 44–45° alone or with an authentic specimen of glucose diethyl mercaptal pentacetate.

The mixture of reducing sugars from the hydrolysed polysaccharide when refluxed with alcoholic aniline for 4 hours yielded a crystalline anilide, m.p. 144–145° alone or in admixture with an authentic specimen of glucose anilide (IRVINE AND GILMORE, 1906). The presence of glucose as a principal constituent sugar of the polysaccharide was thereby confirmed; it was not possible, by absorption on activated alumina, to identify another sugar from the mother liquors of the glucose anilide.

In an endeavour to ascertain whether the glucose residues in the polysaccharide were linked in the (1:4) positions as in glycogen or starch, specimens of the polysaccharide were incubated at 35° C with an active β-amylase preparation from soya beans. There was no generation of reducing sugars as measured by the Schaffer-Hartman technique, and therefore it seems probable that the polysaccharide is different from the glycogen or starch type of structure. Moreover, it did not give a reddish brown colour with iodine in contrast to glycogen.

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Its properties are being compared with those of dextrans synthesized by various micro-organisms.

BIOLOGICAL PROPERTIES

Skin reactions. Polysaccharide II (original preparation # 85) when tested intradermally on patients highly sensitive to PPD-S, gave no skin reaction even when administered in a dosage of 0.05 mg, which is ten times the usual second dose of the tuberculin. See Table II.

TABLE II

SKIN REACTIONS WITH POLYSACCHARIDE II

Patient No.	Area in millimeters of Skin reaction to	
	0.00002 mg PPD-S	0.05 mg Polysaccharide 85
1	36 × 29 × 3	negative
2	24 × 26 × 3	,,
3	37 × 33 × 3	,,
4	21 × 18 × 2	,,
5	12 × 14 × 2	,,
6	14 × 17 × 2	,,
7	35 × 29 × 3	,,
8	31 × 26 × 3	,,
9	24 × 22 × 3	,,
10	17 × 13 × 2	,,
11	48 × 43 × 3	,,
12	15 × 19 × 2	,,
13	15 × 12 × 2	,,

Precipitin titres. High precipitin titres were obtained by adding the Polysaccharide II preparations (# 85 and # 92), to the horse antiserum A 5807, from a horse immunized over a long period of time with large quantities of dead tubercle bacilli in the SHARP AND DOHME laboratories. (See bottom of Table III). This horse serum has also given high titres with Polysaccharide I (SEIBERT, 1932, 1944; MASUCCI, MCALPINE AND GLENN, 1931), as well as with polysaccharide fractions isolated from the tubercle bacillus (HEIDELBERGER AND MENZEL, 1932, 1937).

Precipitin titres, even as high as 1:50000, were also obtained by using Polysaccharide II with most tuberculous rabbit sera tested, with sera from rabbits vaccinated with B.C.G, and with a few human tuberculous sera (see Table III). The amount of precipitate always was slight with these sera even though some of the titres were high.

Six normal rabbits were sensitized with the original polysaccharide preparation # 85 by eleven weekly intracutaneous injections of 10 mg each. Their sera then were shown to contain antibodies for Polysaccharide II (see Table III) thus demonstrating its antigenicity.

Immunization. Six rabbits were given eight intracutaneous injections each of 10 mg. Polysaccharide II (preparation # 85) at weekly intervals and then three successive intraperitoneal injection of the same amount of the polysaccharide to which 10 mg of aluminium hydroxide had been added. The first injection elicited some local reaction evident after 24 hours following the test, and with successive injections the reactions

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TABLE III

PRECIPITIN TITRES WITH POLYSACCHARIDE II

Sera from	Name	Condition	Precipitin titrin with Polysaccharide # 85	# 92
Rabbit	108	Normal	0	
,,	39	,,	0	
,,	RB 31	,,		0
Rabbit	113	Sensitized with Polys. 85	50000	
,,	109	,, ,, ,, 85	10000	
,,	K 70	,, ,, ,, 85	5000	
,,	112	,, ,, ,, 85	5000	
,,	121	,, ,, ,, 85	5000	
,,	37	,, ,, ,, 85	2000	
Rabbit	40	Spontaneous infection	10000	100000
Rabbit	80	Tuberculous	50000	—
,,	81	,,	50000	0
,,	171	,,	50000	—
,,	172	,,	20000	—
,,	40	,,	10000	—
,,	41	,,	10000	20000
,,	114	,,	4000	10000
,,	115	,,	4000	1000
,,	99	,,	4000	2000
,,	120	,,	20000	2000
,,	108	,,	0	0
,,	110	,,	0	2000
,,	96	,,	—	0
,,	105	,,	—	5000?
,,	109	,,	—	0
,,	103	,,	—	0
Rabbit	141	Vaccinated with B.C.G.	2000	
,,	121	,, ,, ,,	5000	
,,	102	,, ,, ,,	10000	
,,	107	,, ,, ,,	0	
Rabbit	143	Sensitized with TPA	—	2000
Human	A.S.	Normal	—	0
,,	H.T.	Normal	0	—
,,	W.B.	Minimal Tuberculosis	—	0
,,	L.W.	Mod. Adv. Tuberculosis	—	50000
,,	F.A.	Far Adv. Tuberculosis	—	20000
,,	R.B.	Far Adv. Tuberculosis	0	
Horse	A 5807	Sensitized with dead TB.	5000000	200000

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became larger, even to practically double the size by the eighth injection. Thus evidence of some local sensitization was seen.

Electrophoretic study of the sera of four of these rabbits did not show a great increase in the gamma globulin fraction during these injections.

One week following the eleventh injection these six rabbits, as well as seven controls that received no previous treatment, and six that had been injected three times with a total of 1.25 mg B.C.G., were inoculated intravenously with 0.00001 mg of live Bovine (RAVENEL) strain tubercle bacilli. They were all allowed to live their natural span of life and then at death autopsied. The amount of tuberculosis was noted by evaluating the percentage of total lung tissue involved with tubercles and also the degree of consolidation by the weight of the lungs. Average results for these criteria of the extent of tuberculosis, as well as the average longevity in each group, were recorded in Table IV and indicate that no significant immunity was developed through sensitization with Polysaccharide II.

TABLE IV

IMMUNIZATION WITH POLYSACCHARIDE II

Method of Treatment	Number Rabbits	Average Longevity in days	Average Tuberculosis in the Lungs	
			Estimated %	Weight H(g)
Controls	7	176	63	52
B.C.G.	6	133	30	46
Polysaccharide # 85	6	175	37	42

DISCUSSION

A polysaccharide with a very large particle weight, designated as glycogen, has been isolated from avian tubercle bacilli by CHARGAFF (CHARGAFF AND MOORE, 1944). It lacked any specific biological activity and had a particle weight of about 13.2 million. LAIDLAW AND DUDLEY (1925) also isolated small amounts of glycogen from tubercle bacilli, of the human type. HEIDELBERGER AND MENZEL (1932, 1937), GOUGH (1932) and HAWORTH, KENT AND STACEY (1948) also mentioned the presence of glycogen among the polysaccharides isolated from tubercle bacilli. The Polysaccharide II described in this paper, like the glycogens, gives an opalescent solution in water, and is mainly constituted of glucose units. Nevertheless it does not give a red brown colour with iodine, it has a high biological specificity, shows some antigenicity, more closely resembles the dextrans, and would, therefore, appear to be a newly identified polysaccharide, manufactured in considerable quantity by certain strains of the tubercle bacillus.

Since the question whether or not anything except proteins can serve as antigens is an important one, some caution is necessary. The Polysaccharide II (preparation # 85) used in these studies for sensitization of the rabbits was shown from its nitrogen content to contain a maximum of 3.3% of possible protein, and, therefore, 3.6 mg would be the total protein given with the polysaccharide during the eleven injections. It is hard to believe that this is sufficient protein to account for the elicitation of the precip-

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itins obtained. Especially is this true, when 15 injections of a Polysaccharide I preparation which contained even more protein impurity, did not elicit any precipitins. Whether the Polysaccharide II is a part of the cell liberated into the medium or whether it is a true metabolite cannot be determined from the present data.

SUMMARY

1. A polyglucosan, with a very low nitrogen content, a relatively large particle weight, and forming opalescent solutions in water has been isolated from several strains of human and bovine type tubercle bacilli.

2. Although it consists mainly of glucose, it is not a glycogen but may be similar to a dextran structurally. It is able to elicit precipitins to itself and it gives a high precipitin titre with a horse antiserum and lower titre with some tuberculous rabbit and human sera.

3. It did not induce a significant immunity in a small number of rabbits, nor did it cause a tuberculin reaction in patients who were sensitive to the protein.

RÉSUMÉ

1. On a isolé de plusieurs souches de bacilles tuberculeux humains et bovins un polyglucosane contenant très peu d'azote et dont les particules sont de poids relativement élevé donnant avec l'eau des solutions opalescentes.

2. Quoiqu'il soit principalement formé de glucose ce n'est pas un glycogène, mais sa structure rappelle quelque peu celle d'un dextrane. Il peut donner naissance à des précipitines et il donne une dilution limite active de précipitine élevée avec l'antisérum de cheval et une dilution-limite active plus faible avec des séra de lapins et humains tuberculeux.

3. Pour un petit nombre de lapins, on n'a pas constaté d'immunité notable; on n'a pas constaté non plus de réactions tuberculines chez les patients qui étaient sensibles à l'action de la protéine.

ZUSAMMENFASSUNG

1. Ein Polyglucosan wurde von mehreren Stämmen menschlicher und tierischer Tuberkelbazillen isoliert. Der Stickstoffgehalt ist sehr niedrig, das Gewicht der Teilchen ist relativ gross und es gibt mit Wasser opaleszierende Lösungen.

2. Obgleich es hauptsächlich aus Glukose besteht, ist es kein Glykogen, sondern hat möglicherweise eine dextranartige Struktur. Es ist fähig in sich selbst Präzipitin zu formen. Es gibt einen hohen Präzipitin-Titer mit Pferdeantiserum und einen niedrigeren Titer mit einigen tuberkulösen Kaninchen- und menschlichen Seren.

3. Bei einigen Kaninchen wurde keine erhebliche Immunität festgestellt; ausserdem sind keine Tuberkulin-Reaktionen bei den Patienten festgestellt worden, die sich gegenüber Protein empfindlich erwiesen hatten.

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