DISSOCIATION AND RECONSTITUTION OF AN ENDOTOXIN

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We reported previously that an endotoxin, which is a complex polysaccharide of high biologic activity obtained from the bacterium Serratia marcescens, showed considerable macromolecular heterogeneity (Mora and Young, 1961). This strongly acidic polysaccharide complex has 80 anionic groups with $pK_a = 2.5$ per 100,000 hypothetical molecular weight unit and contains 10% bound lipid (personal communication from Merler and Saroff). Since sedimentation experiments in the partition cell (Mora and Young, 1961) and other recent work (Ribi et al., 1962) indicated that for certain biological activities of endotoxin a relatively large size (>10S) is necessary, we examined the relation of one biological property, the tumor-damaging potency, to the sedimentation behavior before and after treatment with sodium lauryl sulfate (SLS). Detergents have been used to dissociate macromolecular complexes into their subunits (cf. Hersh and Schachman, 1958, Stellwagen and Schachman, 1962).

We found that SLS dissociated the polysaccharide complex into small units with concomitant loss of biological activity; when the SLS was removed the macromolecular complex reaggregated and the biological activity reappeared.

The endotoxin was a demineralized sample from S. marcescens polysaccharide (Lot No. P45) used in previous studies in this Laboratory (O'Malley, et al., 1962, Oroszlan, et al., 1963). Sodium lauryl sulfate U.S.P. (Duponol C) was used without purification. The induction of hemorrhagic necrosis in mouse Sarcoma 37 was the bioassay employed (cf. Mora and Young, 1961). Sedimentation velocity experiments were conducted at constant temperature (17°C-20°C) with rotor speeds of 59,780 rpm, using schlieren optics. The observed sedimentation coefficients were corrected to values corresponding to a solvent with the viscosity and den-

sity of water at 20°C($s_{20,w}$). The antiserum used was prepared by immunization of rabbits with heat-killed cells of Serratia marcescens. Immunological tests were carried out with the Ouchterlony technique of double diffusion in agar gel (Kabat and Mayer, 1961).

In the first series of experiments, to 1% solutions of endotoxin in pyrogen-free saline, SLS was added in amounts equivalent to the endotoxin, and the solutions adjusted to various pH values. Alternatively, SLS (1%) was first dissolved in saline and the polysaccharide then added in equivalent amounts (as in experiments at pH 4.1 and pH 8.0 in Table I). The mixtures were incubated either at 37°C, or kept at room temperature, for 1 hour. Then aliquots of each mixture were diluted with pyrogen-free saline to give graded doses of endotoxin (2.5-15 µg/0.5 ml/mouse) and bioassayed immediately. Appropriate controls (endotoxin without SLS) were incubated and bioassayed similarly. From another aliquot of each solution, including the controls, the polysaccharide was precipitated with ethanol (66% v/v). This served the purpose of separating the polysaccharide from the detergent in the mixtures. The recovered endotoxin was then dissolved in pyrogen-free saline and bioassayed at a dose level equivalent to 15 µg per mouse of the original endotoxin. In separate bioassays it was ascertained that SLS alone, in doses up to 250 µg/mouse, did not induce tumor damage.

The results (Table I) show that after SLS treatment the tumor-damaging activity of potent endotoxin diminished. At pH 4.1, even without SLS, the biological activity of endotoxin was lost during incubation at 37°C (cf. Hartwell et al., 1943); at pH 9.6 there was some loss of biological activity in the recovered endotoxin. However, at other pH values when the detergent was removed after incubation, the tumor-damaging potency was substantially restored.

In double diffusion experiments in agar (pH 7.4), the endotoxin gave two lines of precipitation: one component diffused more rapidly (appeared closer to the center well, containing the antiserum), and the other component diffused more slowly. Under identical conditions, endotoxin treated with SLS displayed a single line of precipitation, which fused with the faster diffusing component of the untreated endotoxin.

TABLE I

Tumor-damaging Potency of Endotoxin After Incubation at Various pH Values in the Presence and Absence of Sodium Lauryl Sulfate (SLS)

Dose µg per mouse	pH 4.1[a]		7.4[b]		8.0[a]		9.6[b]	
	No SLS	SLS	No SLS	SLS	No SLS	SLS	No SLS	SLS
15	1[c]	0	10	3	10	0	10	3
10	0	0	8	3	7	0	7	3
5	-	-	4	1	-	-	6	2
2.5	-	-	1	1	-	-	4	0
15[d]	0	3	10	7	9	7	6	4

[a]Incubated at 37°C for 1 hour.--[b]Incubated at room temperature for 1 hour.--[c]The figures represent the number of mice with damaged tumors out of 10 mice tested.--[d]Recovered endotoxin--See Text.

We devised a method (Oroszlan and Mora, in preparation) for fractionating this endotoxin, into two major components, by cesium chloride density gradient centrifugation. One component was recovered in low density range; this fraction, in sedimentation velocity experiments in phosphate (0.02 M) buffered saline-solution (pH 7.2), showed relatively large amounts of fast sedimenting material (15.8 S, upper left drawing in Fig. 1). It also had higher tumor-damaging activity than the original unfractionated endotoxin; e.g. the values for the dose which damaged the tumors in 50% of the animals (ED_{50}) were 1 and 5 µg/mouse respectively. This component is designated as "active fraction". The higher density fraction consisted almost completely of slow sedimenting component (3.1 S, lower left drawing of Fig. 1); it had a much lower tumor-damaging activity ($ED_{50} > 50$/mouse) and is designated as "inactive fraction". Agar diffusion experiments showed increased amounts of the slowly diffusing component in the active fraction; the inactive fraction consisted mainly of the rapidly diffusing component.

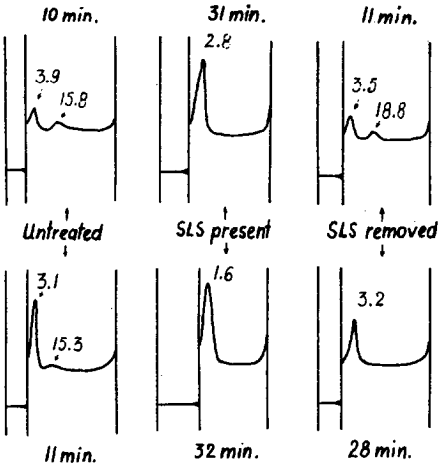

Fig. 1. Sedimentation of the endotoxin fractions after various treatments. Upper row: active fraction; lower row: inactive fraction. The figures are copies of schlieren patterns of 0.5% solutions of the endotoxin fractions with or without SLS (0.5%) after various lengths of time at 59,780 rpm. Numbers near the peaks are sedimentation coefficients in Svedberg units as explained in the text.

We next investigated the effect of SLS on these two fractions. The changes in the sedimentation velocity pattern are summarized in Fig. 1. The results indicate the dissociation of the active fraction after SLS treatment into smaller particles (2.8 S), with loss of biological activity (see Table II); removal of the detergent led to reconstitution of the original components with

TABLE II

The Effect of Sodium Lauryl Sulfate on the Tumor-Damaging Activity of the Active Fraction of Endotoxin Separated by Density Gradient Centrifugation

Sample[a]	Dose µg/mouse		
	10	5	2.5
Untreated	9/9[b]	8/9	7/9
SLS present	3/9	1/9	1/9
SLS removed[c]	8/10	5/9	5/9

[a] 0.5% solutions of active fraction in phosphate-buffered saline, pH 7.2, in the presence or absence of SLS (0.5%) were incubated at 37°C for 1 hour.
[b] The ratios represent the number of mice with damaged tumors over the total number of mice tested.
[c] By alcohol-precipitation of the endotoxin. See text.

concomitant restoration of tumor-damaging potency. In the case of the inactive fraction a 1.6 S component was observed in the presence of SLS; after removal of the SLS it had 3.2 S, and no detectible tumor-damaging activity.

Since the sedimentation coefficients of the active and inactive fractions in the presence of SLS showed small difference (2.8 and 1.6 S respectively), we might assume that the original endotoxin preparation consisted of at least two kinds of "subunits" with different molecular size. Apparently a "subunit" of about 2.8 S of the active fraction, is responsible for the tumor-damaging activity but only in its aggregated form (15-18 S). In contrast, the dissociated particles of the inactive fraction (1.6 S) have the size of the haptenic polysaccharides obtained by acid hydrolysis (Ribi et al., 1962). Although SLS may influence the sedimentation through other effects (cf. Hersh and Schachman, 1958), the relatively sharp sedimentation boundaries in SLS (middle drawings in Fig. 1) make the above assumptions plausible.

These investigations open ways of identifying the macromolecular subunits of endotoxins and illustrate that secondary forces, most probably hydrophobic bonds, are holding together the subunits in the biologically active form.

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