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REVERSIBLE INACTIVATION OF AN ENDOTOXIN BY INTRACELLULAR PROTEIN*

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Abstract—The endotoxic polysaccharide from *Serratia marcescens*, which induced hemorrhage and necrosis in mouse sarcoma 37, was inactivated *in vitro* by extracts of intact cells of rabbit liver. The inactivating potency was associated with the basic protein fraction which was isolated with the aid of charged derivatives of synthetic polyglucose. Macromolecular interaction of the cationic proteins with the anionic endotoxic polysaccharide was demonstrated by zone electrophoresis and metachromasia. Polyglucose sulfate, a stronger polyanion than the endotoxic polysaccharide itself, prevented the inactivation. Since it also restored the original tumor-damaging activity, it was concluded that inactivation was due to a reversible macromolecular interaction of the anionic endotoxic polysaccharide with the cationic liver cell proteins. Cationic derivatives of synthetic polyglucose also inactivated the endotoxin by a similar mechanism.

IT HAS been reported by several authors that endotoxins derived from gram-negative bacteria are inactivated by incubation with mammalian plasma or serum[1–8] or with tissue homogenates.[9] The conditions employed by various investigators in studies of this phenomenon differed widely; this has made it difficult to correlate the findings and to understand the nature of the interaction leading to the inactivation, the attending physicochemical changes, and whether more than one factor is involved. The humoral agent has been shown to be different from complement, properdin, or antibody,[4] and it has been suggested that an enzymatic degradation of the endotoxic polysaccharide might be responsible for the inactivation.[10] The nature of the reaction products, however, remains to be described.

Cationic macromolecules, devoid of enzymatic activity, are also capable of altering some of the biological properties of endotoxins.[11, 12] It has been shown that cationic proteins decreased the pyrogenic and tumor-damaging potency of the anionic endotoxic polysaccharide, apparently through electrostatic interaction of the oppositely charged marcromolecules.[13]

This communication describes the isolation of intracellular material, which is capable of inactivating endotoxin *in vitro*, and the study of the mechanism involved. The induction of hemorrhage and necrosis in transplanted solid tumors in mice by microgram quantities of endotoxin was chosen as the bioassay for measuring the degree of inactivation. Rabbit liver was selected as the tissue from which to isolate host factors because it is an excellent source of high-potency material.[9] The diffusate

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from intact liver cells was fractionated with synthetic polyelectrolytes:[14, 15] charged derivatives of synthetic polyglucose. The cationic proteins isolated in this manner were highly effective in inactivating the tumor-damaging activity of endotoxin and provided a partially purified system for the study of the physicochemical changes paralleling this inactivation. It was found that the endotoxin can be inactivated also by cationic synthetic polysaccharides.

MATERIALS AND METHODS

Endotoxin. The endotoxic polysaccharide used was a sample from lot P-45 prepared by A. Perrault and M. J. Shear of this laboratory from *Serratia marcescens* with a modification of an earlier procedure.[16] The polysaccharide was deionized by passage through a mixed-bed column of Dowex-50 and Dowex-1 ion-exchange resins.[17] This preparation behaved as a relatively strong acid and had 75 to 85 anionic groups, with a pK_a of 2·4 to 2·6, per 100,000 molecular weight unit.[18] It had a low solubility in water and contained 1·2% N, 0·3% P, 44% reducing sugars, and about 10% lipid.[19] The ED_{50} for tumor damage (i.e. the minimal dose inducing damage in the tumors of 50% of the animals) was about 5 μg/mouse.

Synthetic polyglucose derivatives. The anionic polyglucose derivative employed was polyglucose sodium sulfate.[20] It contained three sulfate groups per anhydro glucose unit and had a number average molecular weight of 55,700 .The cationic derivatives of polyglucose[21] were: a tertiary amine, 3-diethylamino-2-(&1)-hydroxy-1-(&2)-propyl ether of polyglucose, N = 4·5%, pK' = 8·5 to 9, degree of substitution per anhydro glucose unit = 0·9; and the quaternary ammonium derivative, poly-{[3-(α-D-glucopyranosyl)-2-hydroxy-1-propyl]-triethyl-ammonium hydroxide}, N = 5·09%, pK' = 9·5, degree of substitution per anhydro glucose unit = 1·6. Both of these cationic derivatives had a number average molecular weight comparable in magnitude to the polyglucose sulfate.

Bioassay. The tumor-damaging potency of the endotoxin was measured in CAF_1 mice of both sexes, weighing 18 to 22 g and bearing 6-day-old intramuscular implants of sarcoma 37, according to the method of Shear *et al.*[22] Twenty-four hours after intraperitoneal injection of a standard volume (0·5 ml/mouse) of solutions containing graded doses of the endotoxin (0 to 20 μg) the mice were killed and the tumors examined for gross hemorrhage and necrosis. Affected tumors were those that showed extensive, freshly induced hemorrhage and necrosis. A minimum of ten mice in each group was used at every dose level of the endotoxin.

Inactivation studies. In general the methods employed by Skarnes *et al.*[4] were followed. The endotoxin was mixed in dilute buffer solutions with the liver cell proteins or other cationic macromolecules, and the mixtures were incubated at 37° or higher for various time intervals. The concentration of the endotoxin in the reaction mixtures was 100 μg/ml (total volume 3 ml) unless otherwise stated. Incubation for 1 hr was sufficient to obtain maximal inactivation. After incubation the mixtures were diluted with pyrogen-free saline to the desired dose level of endotoxin and immediately bioassayed for tumor-damaging potency. Those mixtures which had a pH higher than the physiological value were adjusted to pH 7·4 immediately

before dilution. Three types of controls were included in each set of experiments: (a) buffer control; (b) endotoxin alone, incubated in the appropriate buffer solution; and (c) the complete reaction mixtures without incubation (prepared just before injection). All experiments were repeated several times. The results reported here are based on data obtained in bioassays in which several thousand mice were employed. The data are presented either in summary form or as illustrative protocols.

Preparation of intact liver cells. New Zealand albino rabbits of both sexes, weighing 2·5 to 3·5 kg, were anesthetized with ether. About 80 ml of blood was withdrawn by cardiac puncture and the animals killed. The liver was perfused immediately *in situ* with cold saline.[23] The organ was then excised and a whole cell preparation made according to the method of Kaltenbach[24] by pressing the tissue through a series of successively smaller mesh screens.

Liver cell extract. Zierler[25] demonstrated that special conditions such as anoxia, elevated potassium level, and 37° temperature alter mammalian cell-membrane permeability and increase the efflux of intracellular material into the surrounding medium. Liver cell extracts with endotoxin-inactivating potency have also been obtained under similar conditions, although the method was reported to be not regularly reproducible.[26] Following the above principle we prepared six different extracts with closely reproducible biological activity.

The procedure was as follows. Whole cells of rabbit liver were suspended in divalent cation-free Ringer's–phosphate medium (0·132 M NaCl, 0·005 M KCl, 0·001 M KH_2PO_4, 0·01 M Na_2HPO_4; pH 7·4; proportion of wet-cell brei to suspending medium = 1:2 w/v) and incubated at 37° for 1 hr while nitrogen gas was bubbled through the suspension. After centrifugation at 35,000 × *g*, at 5° the clear supernatant was dialyzed in Visking cellophane tubing against cold distilled water for 48 hr. During dialysis about 88% of the total nondialyzable solutes (8 to 10 mg/ml) was precipitated. The supernatant was lyophilized; substantially all of the original endotoxin-inactivating potency was recovered in this latter water-soluble fraction

TABLE 1. ENDOTOXIN-INACTIVATING POTENCY OF CRUDE LIVER CELL EXTRACT AND OF ITS FRACTIONS OBTAINED BY DIALYSIS

Liver cell extract	Nondialyzable solids		Tumor damage†
	% of total	Amt. added to incubation mixture* (mg/ml)	
Before dialysis	100	3·4	3/10
After dialysis			
Supernatant	11·8	0·4	3/10
Precipitate	88·2	3·0	8/10
Control (20 μg endotoxin/mouse)			10/10

* Incubation in divalent cation-free Ringer's–phosphate medium, pH 7·4, 37°, 1 hr. The mixtures, containing 0·2 mg endotoxin/ml and the given amounts of samples, were diluted for bioassay to the dose level of control.

† Number of mice with damaged tumors/total number of mice tested.

(Table 1). By weight this represented about 0·3% of the original wet tissue and about 12% of the crude live cell extract. This was the type of preparation used in further experiments (referred to as "liver cell extract," LCE).

Paper chromatography. Paper chromatography of the bacterial polysaccharide preparation was carried out by the ascending technique on Whatman No. 1 filter paper at room temperature. The solvent was diluted propanol[27, 28] buffered at various pH values; 45 to 75 μg endotoxin in the appropriate buffer was applied on the paper. The filterpaper strips were stained with periodic acid-Schiff (PAS) reagent.[29]

Zone electrophoresis. Electrophoretic analysis of the bacterial polysaccharide and of the liver cell proteins or their mixtures after incubation was carried out either on cellulose acetate membrane[30] or on glass-fiber paper. When the latter support was used in alkaline borate buffer the two ends of the strips were not immersed directly in the electrolyte solution but were in contact with it through a cellophane membrane[31] in order to minimize electroendosmosis. Toluidine blue[32] and/or PAS reagent[29] were used on both supporting media to detect the polysaccharide spots. In some instances the glass-fibre paper was sprayed with *p*-anisidine reagent.[33] Light green and/or nigrosin were used for staining the proteins.[30]

Ultracentrifugation. Ultracentrifugation was performed in the Spinco model E ultracentrifuge (Schlieren optics). Before centrifugation, suspensions of the endotoxic polysaccharide in Ringer's-phosphate medium were adjusted to pH 9·5 in order to solubilize the polysaccharide complex, the pH of the solution was then lowered immediately to 7·4. All centrifugations were carried out in this medium at 1% polysaccharide content. Sedimentation coefficients are given in Svedberg units after correction to water at 20° with the exception that no attempt was made to estimate the partial specific volume term.

Metachromasia. The metachromatic dye used was toluidine blue 0, CI No. 925 (National Aniline Division). The absorption spectra of dye solutions and of the dye–polyelectrolyte mixtures (the bacterial polysaccharide with or without proteins) were taken in the Beckman DU spectrophotometer.

Bacterial polysaccharide concentration. This was determined chemically by the anthrone method.[34] *Reducing sugars* were determined by a modified Hagedorn–Jensen method,[35] and *nitrogen* by Nesslerization.[36]

RESULTS

I. Separation of the endotoxin-inactivating component from liver cell extract by charged synthetic polyglucose derivatives

The liver cell extract was further fractionated with the aid of charged synthetic polyglucose derivatives. Polyglucose sulfate, a strong polyanion, precipitated the endotoxin-inactivating material. The precipitate obtained with the polycation, 3-diethylamino-2-(&1)-hydroxy-1-(&2)-propyl ether of polyglucose, was inactive, (the most anionic components of the liver cell extract were precipitated in this step), and all the activity remained in the supernatant.

The details of our procedure were as follows (cf. Table 2).

TABLE 2. SEPARATION OF ENDOTOXIN-INACTIVATING COMPONENT OF LIVER CELL EXTRACT WITH CHARGED SYNTHETIC POLYSACCHARIDES

Polyglucose sulfate ($PGSO_3^-$) Endotoxin-inactivating potency*			Control: 10 μg endotoxin/mouse 20/30†	$PG....\overset{+}{N}Et_2$‡ Endotoxin-inactivating potency*		
Inactivation (%)	Tumor damage†	(μg/ml)		(μg/ml)	Tumor damage†	Inactivation (%)
80	4/30	100	Liver Cell Extract	100	4/30	80
55	6/20	50		50	6/20	55
			Add $PGSO_3^-$; pH 4	Add $PG....\overset{+}{N}Et_2$; pH 8·2		
			Precipitate P_1 Diss. at pH 7 Add IR-45 Dial., Lyophil. Yield: 6%	*Supernatant* S_1' Add IRC-50; pH 7 Dial., Lyophil. Yield: 47% LCP		
85	3/30	100	*Supernatant* S_1 Lower pH to 1	100	0/30	100
				50	1/20	93
			Precipitate P_2 Diss. at pH 7 Add IR-45 Dial., Lyophil. Yield: 36%	*Precipitate* P_1' Diss. at pH 6 Add $PGSO_3^-$; pH 5		
75	5/30	100	*Supernatant* S_2 Add IR-45 Dial., Lyophil. Yield: 48%	*Supernatant* S_2' Add IR-45 Dial., Lyophil. Yield: 58%		
30	14/30	100		100	21/30	0
				Precipitate P_2' ($PGSO_3^-$–$PG....NEt_2$ complex)		

* Incubation with 100 μg endotoxin per ml of divalent cation-free Ringer's-phosphate, pH 7.4, 37°, 3 hr. Diluted to the dose level of control.
† Number of mice with damaged tumors per total number of mice tested.
‡ 3-Dimethylamino-2-(&1)—hydroxy-1-(&2)-propyl polyglucose.

1. Fractionation with polyglucose sulfate (left side of Table 2). to an aliquot of LCE dissolved in pyrogen-free distilled water (0·33%, w/v; pH 6·5) sodium polyglucose sulfate was added in a ratio of 1:1. The pH of the solution was lowered with dilute sulfuric acid to 4, the point where the first precipitate (P_1) was formed; the supernatant (S_1) was kept for further fractionation. The precipitate (P_1) was suspended in distilled water and was then dissociated by adjusting the pH to 7 with dilute sodium hydroxide. Amberlite IR-45 (OH) was added to the solution (10 mg/ml) to remove the polyglucose sulfate. The decanted supernatant was dialyzed and lyophilized, 6% of the original extract was recovered in this fraction which almost completely inactivated the endotoxin at a ratio of 1:1, w/w, as shown in Table 2. On lowering the pH of the supernatant (S_1) to pH 1, further precipitation took place with polyglucose sulfate. This precipitate (P_2) was treated in a similar manner to precipitat-P_1. The material, recovered in a yield of 36% of the original, showed somewhat less activity than P_1. The supernatant (S_2) of this second precipitation step, after removal of the polyglucose sulfate with IR-45, was also dialyzed and freeze-dried (yield = 48%). This fraction still contained some activity; incubation with the endotoxin at a ratio of 1:1, w/w, resulted in a 30% inactivation of the latter. Although polyglucose sulfate was capable of precipitating factors which inactivated the endotoxin, the activity on a weight basis of the fractions as compared with that of the original extract was not increased. Thus an efficient separation of the active components was not obtained with this polyanion.

2. Fractionation with the cationic polyglucose derivative (right side of Table 2): in early experiments 3-diethyl-amino-2-(&1)-hydroxy-1-(&2)-propyl ether of polyglucose was added in a ratio of 1:1 to an aliquot of the LCE dissolved in pyrogen-free distilled water (0·33%, w/v; pH 6·5). The final pH of this solution was 9·8. When the pH was lowered with dilute hydrochloric acid to pH 8·2, a heavy precipitate (P_1) was formed. It was dissolved in distilled water adjusted to pH 6. Polyglucose sulfate, in amount equivalent to the polyglucose amine, was added to this solution and the pH lowered to 5. At this pH the polyglucose amine formed an insoluble complex with the added polyglucose sulfate, leaving in solution the amine-precipitated components of the cell extract. This supernatant, after removal of the traces of polyglucose sulfate with IR-45, was dialyzed and lyophilized; it showed no endotoxin-inactivating potency.

To the supernatant (S_1') Amberlite IRC-50(H) was added (10 mg/ml) to remove traces of the polyglucose amine. When the pH reached neutrality, the solution was decanted, dialyzed against cold distilled water, and freeze dried. An active material, designated as live cell protein (LCP), was obtained in a yield of 47%. This preparation was capable of completely inactivating the endotoxin at a ratio of 1:2 (50 μg liver cell protein/ml incubated with 100 μg endotoxin/ml; Table 2). Thus it was about two times as active as the initial liver cell extract.

Polyglucose amine was used to prepare larger amounts of active liver cell protein. In these experiments a somewhat modified procedure was used: the cationic polyglucose amine (50 mg/ml) was added, dropwise with stirring, directly to the supernatant of the dialyzed cell extract (140 ml, obtained from two rabbit livers) until no further precipitate was formed (final pH 8·2 to 8·5). The amount of polyglucose amine needed was about 100 mg. After centrifugation and decanting, the supernatant was brought back to neutrality with IRC-50(H), dialyzed in the cold, and finally lyophilized.

The active material (LCP) was similar in biological and chemical properties to that obtained by the previous method.

In Fig. 1 the absorption spectra of the liver cell extract and the liver cell proteins are shown. The ratios of absorbancies, $A_{280\ m\mu}/A_{260\ m\mu}$, were found to be 0·72 and 1·2 respectively. The N content of the preparations (LCP) ranged between 14·0 and

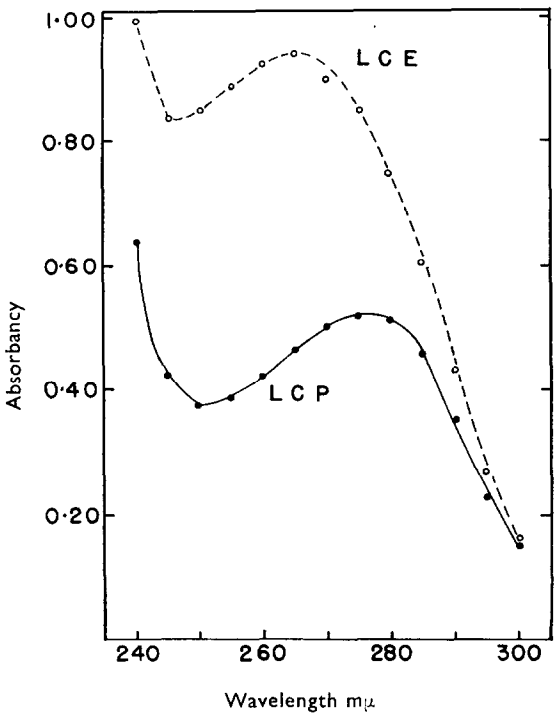

FIG. 1. Absorption spectra. LCE: liver cell extract (0·5 mg/ml; $A_{280}/A_{260} = 0·72$). LCP: liver cell protein (0·5 mg/ml; $A_{280}/A_{260} = 1·2$). Divalent cation-free Ringer's–phosphate solution, pH 7·4, 1-cm lignt path.

14·5%. The qualitative protein tests, including the Sakaguchi reaction for guanidyl groups, were positive. These partially purified protein preparations could be resolved into three major components which migrated towards the cathode during electrophoresis on cellulose acetate strips (Michaelis buffer, pH 8·6, I 0·1, 0·4 mA/cm; see Fig. 4).

II. The nature of the inactivation of endotoxin by liver cell protein

Preparations obtained as described above were then employed in study of the mechanism by which they inactivated endotoxin.

Effect of pH and temperature. It has been shown that the inactivation of endotoxin both by plasma[4, 10] and by liver homogenates[9] proceeds over a wide pH range, being optimal on the alkaline side, between pH 9 and 10. In our experiments the endotoxin (100 μg/ml) was incubated at 37° for 1 hr, with the liver cell protein (100 μg/ml) in

divalent cation-free Ringer's–phosphate medium adjusted to various pH values from 7·4 to 11. Between pH 7·4 and 10, complete suppression of tumor-damaging potency was obtained; at pH 11 no inactivation took place.

Incubation at higher temperatures (i.e. 56°, 70°, and 90°) also led to complete inactivation. However, when the liver cell protein fraction was heated alone at these temperatures, even at 56° it coagulated rapidly. This was accompanied by loss of endotoxin-inactivating potency. Apparently the bacterial polysaccharide in the mixtures prevented the heat coagulation of liver cell proteins.

Complete inactivation was also obtained at 37° in 0·1 M Michaelis-buffer, pH 8·6, and 0·05 M borate buffer, pH 9·1.

Effect of divalent cation and of ionic strength. It has been reported that Ca^{2+}, while strongly inhibiting the inactivation of endotoxin by plasma,[4] does not interfere with the action of liver homogenates.[9]

We investigated the effect of C^{2+} on the liver cell protein as well as on the endotoxin-inactivating fraction of rat plasma separated by continuous paper curtain electrophoresis.[37] The endotoxin was incubated in a concentration of 100 μg/ml with 100 μg LCP/ml, or with 200 μg plasma fraction per ml in divalent cation-free Ringer's–phosphate medium, pH 7·4, at 37° for 1 hr, in the presence of various amounts of $CaCl_2$. Under the above conditions, without the divalent cation, the endotoxin was completely inactivated by the agents for both sources. Although Ca^{2+} in low concentration (0·0025 M $CaCl_2$) completely suppressed the inactivation of endotoxin by the plasma fraction, it showed only a very slight or no inhibitory effect on the action of liver cell proteins at a concentration ten times higher (0·025 M $CaCl_2$).

The results given in Table 3 show the inhibitory effect of sodium chloride; reduction of endotoxin-inactivating ability of liver cell proteins was noted at the higher salt concentrations.

TABLE 3. THE EFFECT OF IONIC STRENGTH ON THE ENDOTOXIN-INACTIVATING POTENCY OF LIVER CELL PROTEIN

100 μg endotoxin/ml incubated with 100 μg liver cell protein/ml in divalent cation-free Ringer's–phosphate medium (pH 7·4, 37°, 1 hr).

Added NaCl M	Inactivation (%)
0	100
0·5	50
1·0	33
2·0	33

Attempts to detect enzymatic breakdown products. It has been suggested that enzymatic degradation of the bacterial polysaccharide might be responsible for its inactivation by the plasma and tissue factors.[9, 10] We therefore attempted to detect reaction products.

The endotoxic polysaccharide was incubated with the liver cell protein fraction for a sufficient duration of time (1 hr) to obtain complete inactivation of tumor-damaging potency. The incubated endotoxin-liver cell protein mixtures and their

dialysates were analyzed for sugars with the anthrone method as well as with a micromethod based on the reduction of ferricyanide. This latter method is sensitive down to the range of 1 to 9 μg of glucose.[35] A dialyzable carbohydrate product or an increase in the reducing power of the mixture could not be found.

Paper chromatography and electrophoresis of the inactive mixtures did not reveal decomposition of the original polysaccharide into smaller units.

The endotoxic polysaccharide was separated at pH 4 (25% propanol in 0·1 M acetate buffer) and at pH 7·5 (25% propanol in 0·05 M phosphate buffer) into two components: one moved almost with the solvent front (the Rf values were 0·89 and 0·95 respectively); the other remained at the place of application. A similar chromatogram was obtained when the biologically inactive mixture of endotoxin and liver cell proteins (100 μg endotoxin/ml incubated with 100 μg LCP/ml in 0·05 M phosphate buffer, pH 7·5, at 37° for 1 hr) was run in propanol–phosphate buffer at pH 7·5; however the Rf value (0·91) of the moving fraction was somewhat lowered. Paper chromatography of the polysaccharide using 25% propanol in 0·1 M borate buffer, pH 9·6, as solvent resulted in one spot just behind the solvent front (Rf = 0·95).

Figure 2 illustrates the electrophoretic pattern on glass-fiber paper in borate buffer, at pH 9·1, of the endotoxin alone and after incubation with the cell protein (LCP). In both cases only two fractions could be detected by staining either with PAS or with *p*-anisidine. However, in the presence of the proteins the migration rate of the very slowly moving component of the endotoxin was visibly altered and was similar to that of the proteins, as ascertained by staining the latter on separate strips. The proteins alone did not move under the conditions employed. This indicated that a complex may have been formed between the slowly migrating component of the bacterial polysaccharide and the liver cell proteins and that this complex remained stable during electrophoresis. In further experiments, upon elution of the separated fractions of the untreated endotoxin, some of the tumor-damaging activity was recovered in the more slowly migrating fraction whereas the faster component was inactive.

Effects of incubation time, of the ratio of endotoxin to liver cell protein, and of added polyglucose sulfate. The results described above suggested that the suppression of the tumor-damaging potency was not due to an enzymatic breakdown of the polysaccharide moiety of the endotoxin. Nevertheless, experiments were carried out to determine whether the inactivation proceeds at all catalytically and whether it is irreversible.

The graph on the left side of Fig. 3 shows the results of an experiment in which a fixed amount of protein (50 μg/ml) was employed. This amount had previously been found capable of inactivating 100 μg endotoxin/ml. An aliquot of the mixture was taken for bioassay every hour; immediately thereafter additional endotoxin was added to the incubation mixture, in the event that the hypothetical "substrate" (the endotoxin) might have been exhausted. As can be seen, the total amount of endotoxin inactivated remained constant in spite of the increased endotoxin concentration and prolonged incubation time. Thus the inactivation did not proceed catalytically, even when excess endotoxin (substrate) was added.

In the next experiment (right graph of Fig. 3) we started with a higher concentration of endotoxin (300 μg/ml) and added, stepwise, a certain amount of protein

(50 μg/ml) at hourly intervals. The dashed line shows the recovery of endotoxin a assayed by its tumor-damaging activity. It can be seen that a ratio of protein to endotoxin of 1:2 was needed, as previously observed, in order to obtain complete inactivation. At this time (after 3-hr incubation) we added to the reaction mixture

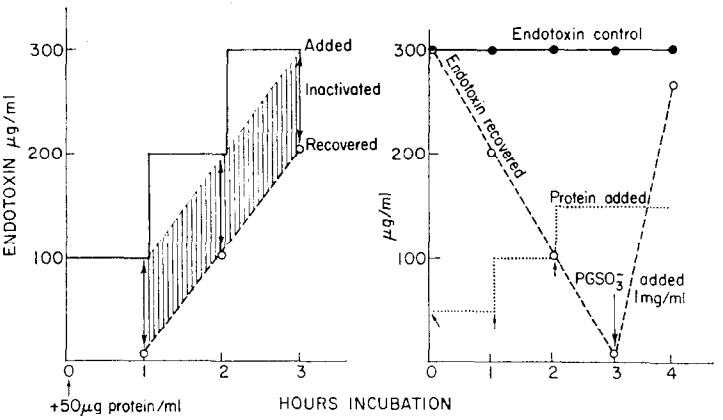

FIG. 3. The effect of incubation time, of the ratio of endotoxin to liver cell protein, and of added polyglucose sulfate on the inactivation of the tumor-damaging potency of endotoxin; see text. Incubation in divalent cation-free Ringer's–phosphate solution, pH 7·4, at 37°. Free endotoxin concentrations after incubation were determined by bioassay and calculated from the recovered relative activities.

polyglucose sulfate, a stronger polyanion than the endotoxic polysaccharide, and incubated the mixture for another hour. Almost all the original activity was recovered, a finding difficult to reconcile with enzymatic degradation of the hypothetical "substrate."

TABLE 4. INACTIVATION OF ENDOTOXIN BY LIVER CELL PROTEINS; THE EFFECT OF POLYGLUCOSE SULFATE ($PGSO_3^-$)

Tumor-damaging potency (15 μg endotoxin/mouse) after incubation* with					
LCP (μg/mouse)	T.D.†	LCP + $PGSO_3^-$ (μg/mouse)	T.D.†	First LCP and then $PGSO_3^-$ (μg/mouse)	T.D.†
0	10/10				
3·75	4/10				
7·5	2/10				
15	0/10	15 + 15	2/10	15 + 30	1/10
		15 + 90	10/10	15 + 90	10/10

* Divalent cation-free Ringer's–phosphate medium, pH 7·4, 37°, 1 hr; 0·5 ml of 3:10 dilutions of the incubated mixtures was injected into each mouse. This contained 15 μg endotoxin and the appropriate doses of the other reactants.

† Tumor damage = number of mice with damaged tumors/total number of mice tested.

In Table 4 further data are given on the effect of polyglucose sulfate. In the first column the inactivation of the tumor-necrotizing potency is shown when the endotoxin was incubated with increasing concentrations of liver cell protein alone. The presence

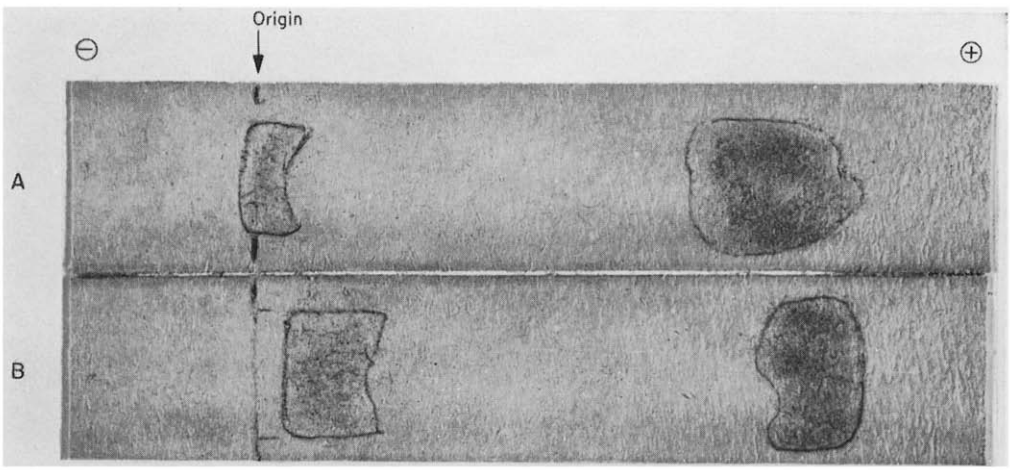

FIG. 2. Zone electrophoresis on glass-fiber paper. A: Endotoxin (60 μg) alone. B: Endotoxin after incubation with liver cell protein (60 μg) at 37° for 1 hr; borate buffer 0·05 M, pH 9·1; run at 4° for 2 hr; 15 V/cm. Staining the strips was performed with PAS reagent. Identical results were obtained when *p*-anisidine reagent was used to detect the spots.

of polyglucose sulfate in excess (second column) prevented the inactivation; it also restored the original activity of the previously inactivated mixture (third column).

Polyglucose sulfate alone, even in much higher concentrations than used in the above inhibition studies, had no synergistic or potentiating effect on the tumor-damaging activity of various lower doses of the endotoxin, as summarized in Table 5.

TABLE 5. THE EFFECT OF POLYGLUCOSE SULFATE ($PGSO_3^-$) ON THE TUMOR-DAMAGING POTENCY OF ENDOTOXIN*

Endotoxin (μg/mouse)	$PGSO_3^-$	Tumor damage†
2·5		3/10
2·5	400	4/10
5·0		5/10
5·0	400	6/10
10·0		8/10
10·0	200	8/10

* Incubation in divatlent cation-free Ringer's–phosphate medium, pH 7·4, 37°, 1 hr; 0·5 ml of 3:10 dilutions of the incubated mixtures were injected into each mouse. This contained the doses given in the table.

† Number of mice with damaged tumors per total number of mice tested.

Thus these results indicated that the inactivation of the tumor-necrotic potency of the endotoxin by the cationic liver proteins was a consequence of reversible interaction of oppositely charged macromolecules and not of an enzymatic breakdown of the endotoxic polysaccharide.

Electrophoresis on cellulose acetate strips. The interaction was also studied by electrophoresis on cellulose acetate; the results are shown in Fig. 4.

The endotoxin, after solubilization at alkaline pH, was resolved at pH 8·6 in barbital buffer into two fractions: a faster migrating, strongly metachromatic fraction, stained with toluidine blue, and a slower migrating fraction which could not be stained well with the basic dye but could be detected by PAS reagent. The tumor-damaging activity of the endotoxin was apparently associated with this latter component.[38] The liver cell proteins alone migrated toward the cathode and were resolved into three major fractions. When the endotoxin–protein mixture was subjected to electrophoresis, after incubation and inactivation, both the protein and the carbohydrate patterns were altered. Some of the proteins now migrated to the anodic side. Staining with PAS reagent revealed protein-bound carbohydrate all over this altered protein pattern with an accumulation in certain places. The metachromatic fraction migrated somewhat more slowly than in the absence of protein, indicating that it was also complexed with the protein but that it dissociated in the electric field.

Metachromasia. An interaction of the anionic polysaccharide with the cationic cell proteins could be demonstrated also by spectrophotometry with the aid of metachromasia.

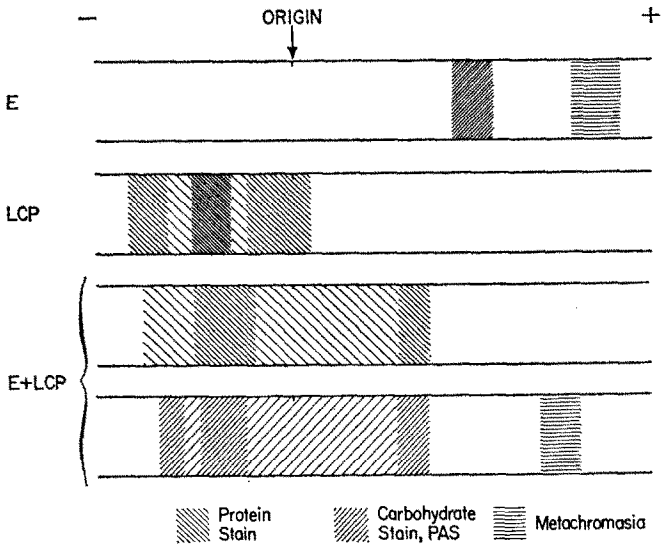

FIG. 4. Schematic illustration of the endotoxin-liver cell protein interaction as studied by electrophoresis on cellulose acetate membranes. E: endotoxin (45 μg). LCP: liver cell protein (45 μg). E + LCP: endotoxin (45 μg) incubated with liver cell protein (45 μg). Samples were incubated at 37° for 3 hr before application. Michaelis buffer pH 8·6, I 0·1, room temperature, 3 hr, 0·4 mA/cm.

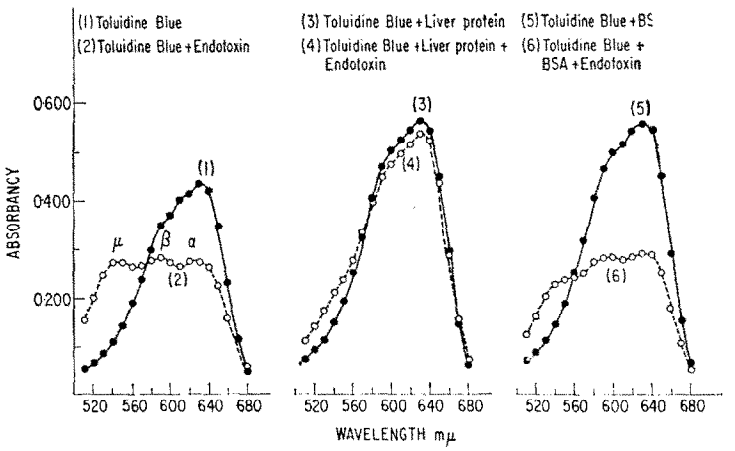

FIG. 5. Absorption spectra of toluidine blue in the presence or absence of endotoxin with or without added proteins; 1:1 diluted divalent cation-free Ringer's–phosphate, pH 7·4, 1-cm light path. (1) Toluidine blue (1·42 μg/ml); (2) toluidine blue + endotoxin (500 μg/ml); (3) toluidine blue + liver cell protein (250 μg/ml); (4) toluidine blue + endotoxin + liver cell protein; (5) toluidine blue + bovine serum albumin (250 μg/ml); (6) toluidine blue + endotoxin + bovine serum albumin.

In Fig. 5 curve 1 shows the absorption spectrum of toluidine blue. In the presence of the anionic bacterial polysaccharide, which binds the basic dye, the metachromatic curve (No. 2) showed three absorption maxima; the color of the solution changed from lblue through purple to reddish violet. In the presence of liver cell proteins (curve 4), this color change did not take place. As can be seen, curve 4 is almost identical with curve 3, the absorption curve of the toluidine blue–protein mixture. However, in the presence of bovine serum albumin which did not inactivate the endotoxin, the metachromatic reaction between the acidic polysaccharide and basic dye was not affected to any appreciable degree, as shown by curves 5 and 6.

FIG. 6. Sedimentation patterns. Top: 1% endotoxin. Middle: endotoxin (1%) with liver cell protein (0·5%) after incubation at 37° for 1 hr. Bottom: 0·5% liver cell protein. Solvent: divalent cation-free Ringer's–phosphate solution, pH 7·4. Pictures were taken 52 min after the maximal speed (59,780 rpm) was reached. Temperature 25°; sedimentation from right to left.

Sedimentation in the ultracentrifuge. The sedimentation patterns are shown in Fig. 6. The polysaccharide (top diagram) showed a major component with 2·3 S and a higher molecular weight component with 6·2 S. A small amount of a faster sedimenting third component (>10 S), probably composed of a whole range of polymers, was also present. A 0·5% solution of the liver cell protein (lowest diagram) exhibited one peak with 2·6 S. When the mixture of the endotoxin and of protein was centrifuged (middle diagram) after incubation for 1 hr at 37°, again two peaks appeared, one with 2·2 S and the other with 3·7 S, and the presence in small quantities of a faster moving third component was also indicated.

III. *Inactivation of the endotoxin by cationic synethetic polyglucose derivatives*

When the endotoxin was incubated with cationic nitrogen-containing derivatives of synthetic polyglucose, under conditions similar to those used for the inactivation by liver cell proteins (Ringer's–phosphate medium, pH 7·4, 37°, 1 hr), the tumor-damaging potency disappeared.

The quaternary ammonium derivative, being a stronger cation than the teritary amine, was found the more effective: smaller amounts of the former were needed in needed in order to obtained complete inactivation. Representative data are given in Table 6. When polyglucose sulfate was added to the inactive mixture of endotoxin

TABLE 6. INACTIVATION OF ENDOTOXIN BY CATIONIC SYNTHETIC POLYGLUCOSE DERIVATIVES, AND THE EFFECT OF POLYGLUCOSE SULFATE ($PGSO_3^-$) ON THIS INTERACTION

Tumor-damaging potency (15 μg endotoxin/mouse) after incubation* with					
PG.....$\overset{+}{N}Et_3OH^-$†		PG.....$\overset{+}{N}Et_2$‡		First PG.....$\overset{+}{N}Et_2$‡ and then with $PGSO_3^-$	
(μg/mouse)	T.D.§	(μg/mouse)	T.D.§	(μg/mouse)	T.D.§
0	10/10	0	10/10		
15	1/10	30	0/10	30 + 60	9/10

* Divalent cation-free Ringer's–phosphate medium pH 7·4, 37°, 1 hr; 0·5 ml of 3:10 dilutions of the incubated mixtures was injected into each mouse. This contained 15 μg endotoxin and the appropriate doses of the other reactants.

† Quaternary ammonium derivative: [poly-{[3-(α-D-glucopyranosyl)-2-hydroxy-l-propyl]-triethylammonium hydroxide}.

‡ Tertiary amine derivative: 3-diethylamino-2-(&1)-hydroxy-1-(&2)-propyl ether of polyglucose.

§ Tumor damage = number of mice with damaged tumors/total number of mice tested.

and the tertiary amine derivative of the polyglucose, and incubated for an additional hour, the original tumor-damaging activity reappeared.

DISCUSSION

The result presented indicate that the material in the liver cell extract responsible for the inactivation of the tumor-necrotizing potency of the endotoxin is associated with the basic protein fraction. This fraction was isolated with the aid of charged synthetic polyglucose derivatives. Electrophoretic analysis provided further evidence for the cationic nature of the protein components of the active fraction.

The inactivation of the tumor-damaging potency of the endotoxin preparation from *S. marcescens* was the result of a reversible interaction of this anionic polysaccharide with the cationic liver cell proteins. Polyglucose sulfate, which contains stronger dissociating anionic groups and has higher negative charge density than the endotoxic polysaccharide,[20] inhibited the inactivation of the endotoxin by the cationic proteins and was also capable of restoring the original activity of the endotoxin after inactivation by protein. Thus the mechanism involved in this inactivation process is similar to that already suggested for the electrostatic interaction of the anionic bacterial polysaccharide with various cationic macromolecules.[13]

However, the finding that our protein preparations from rabbit liver possess high

capacity for inactivation of endotoxin by complexing with it reversibly, does not necessarily conflict with the postulated role of enzymes in the inactivation with plasma[10] or the tissue homogenates.[9] It may well be that there are present in the mammal several factors capable, by different mechanisms, of inactivating endotoxin.

The suppression of the tumor-damaging potency of the endotoxin by interaction with cationic polyglucose derivatives further demonstrated the importance of electrostatic forces in the inactivation of endotoxin.

This concept was corroborated by observations showing that increasing ionic strength markedly decreased the capacity of liver cell proteins to interfere with the biological activity of endotoxin. Also, the metachromatic reaction which took place between toluidine blue and the acidic polysaccharide, and which is known[39] to be caused by the combination of the cationic dye with the anionic groups of the chromotrope (in our case the endotoxic polysaccharide), did not occur in the presence of liver cell proteins. The cationic liver cell protein apparently prevented the binding of toluidine blue to the polysaccharide by itself combining with the same anionic groups of the latter. Similar competition between dye molecules and various proteins was observed in studies of the metachromatic properties of mucopolysaccharides.[40, 41] However, it should be noted that the inhibition of metachromasia and the inactivation of the endotoxin by liver cell proteins are not completely parallel processes: the former occurs at room temperature and also immediately after the addition of the proteins to the reaction mixture, while the latter is time dependent and takes place readily only at body temperature—i.e. 37°. Furthermore, the strongly metachromatic fraction of the endotoxin is apparently an inactive component.[38]

The results obtained when the interaction of the polysaccharide with liver cell proteins was studied by electrophoresis might suggest the formation of relatively firm complexes, stable in the electric field, and probably involving a family of various molecular species. We should not overlook the possible participation of other than simple coulombic binding forces in the inactivation process. Besides the electrostatic attraction, other short-range forces, such as hydrogen bonds and specific structural complementarity, have been postulated in the formation of complexes between anionic polysaccharides and proteins.[42, 43]

Nevertheless, on the basis of our data it is clear that electrostatic forces are important in the inactivation, by the cationic intracellular proteins, of the tumor-damaging potency of the anionic endotoxic polysaccharide.

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