TABLE I. Effect of 8 Days of Starvation on Cardiovascular Parameters of Male Chickens, 5 Months (Trial 1) and 15 Months (Trial 2) of Age.

Measurement	Trial 1		Trial 2	
	Pre-starved	Starved	Pre-starved	Starved
(Number of birds)	(10)	(9)	(9)	(6)
Body wt, g	1961 ± 58†	1356 ± 74*	2425 ± 58	1893 ± 62*
Systolic BP, mm Hg	224 ± 9.7	139 ± 9.2*	206 ± 5.3	158 ± 5.1*
Diastolic BP, " "	168 ± 6.7	124 ± 6.7*	158 ± 3.7	132 ± 4.6*
Pulse pressure, " "	56 ± 8.1	14 ± 3.1*	48 ± 5.0	26 ± 2.1*
Mean BP, " "	189 ± 7.0	130 ± 7.6*	176 ± 3.3	142 ± 4.7*
Heart rate, per min.	342 ± 8.0	297 ± 23	303 ± 12.0	235 ± 13.8
Cardiac output				
ml/min.	330 ± 19	126 ± 22*	493 ± 27	327 ± 35*
ml/min./kg	170 ± 11	88 ± 10*	202 ± 8	173 ± 16
Stroke volume, ml	.97 ± 11	.40 ± .09*	1.65 ± .12	1.39 ± .11
Total periph. resistance				
TPR units	.59 ± .03	1.19 ± .13*	.37 ± .03	.45 ± .05
TPR units/kg	1.16 ± .08	1.65 ± .10*	.89 ± .05	.85 ± .06
Hematocrit	39 ± .90	44 ± .96*	44 ± 1.2	45 ± 2.7

* Difference between pre-starved and starved groups significant at .05 level.
† Mean ± S.E.

that starvation and not Vit. B_1 deficiency *per se* causes bradycardia in chickens.

Summary. Acute starvation of chickens produces a drop in heart rate, blood pressure and cardiac output. The decline in heart rate, which is influenced by excitement and handling of the bird, may be due to enhanced vagal tone.

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Preparation and Properties of an Aluminum Citrate-Endotoxin Complex from *Salmonella enteritidis*. (27966)

W. T. HASKINS, E. RIBI, M. LANDY, R. L. ANACKER AND K. C. MILNER
(Introduced by J. J. Munoz)
U. S. Dept. of HEW, Public Health Service, Nat. Inst. Health, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Mont.

Aqueous ether extracts containing the endotoxin of *Salmonella enteritidis* have previously been processed in various ways in order to reduce their content of protein and lipid. Treatments included fractional precipitation with ethanol and salts, extraction with boiling mixtures of chloroform-ethanol, and extraction with aqueous phenol. Resulting preparations contained as little as 0.4-0.5% nitrogen and about 3-4% total fatty acids (1).

Since these procedures were tedious and time consuming, other means were sought for preparing endotoxin with low nitrogen and fatty acid contents. In this study the nitrogen content was reduced by treating aqueous

TABLE I. Nitrogen Analyses of Aqueous Ether Extracted Endotoxin* from *S. enteritidis* Refined by Treatment with Phenol-Water and Centrifugation.

Preparation	Before centrifugation nitrogen (%)	After centrifugation nitrogen (%)	Hexosamine nitrogen (%)
1	.70	.47	.27
2	.73	.45	.26
3	.62	.58	.29
4	.67	.46	.27
5	.52	.42	.23
6	.57	.55	.27

* Conventional aqueous ether extracted endotoxin contains 3-6% nitrogen.

ether extracted endotoxin directly with phenol water. A single extraction followed by high speed centrifugation reduced nitrogen content from 3-6% to 0.4-0.6%. Up to one-half of the total fatty acids was also removed.

In the second phase of this study we sought to remove lipid from endotoxin by reductive cleavage of ester and amide linkages of the fatty acids with lithium aluminum hydride. Although little or no reduction of firmly bound fatty acid was achieved, an aluminum citrate-endotoxin complex having the desirable features of high biological potency and a rapid rate of solution in water was obtained.

Experimental and results. 1. *Preparation of endotoxin with low nitrogen content.* Rapid and efficient reduction of nitrogen content was achieved as follows: A 2% aqueous solution of endotoxin from *S. enteritidis,* referred to as conventional aqueous ether extract (68% ethanol precipitated, dialyzed aqueous ether extract(1)), was stirred with an equal volume of liquefied phenol at 65°C for 30 minutes. The mixture was chilled in ice water and the phenol and water phases separated by centrifugation at 1600 × *g* for 10 minutes. The aqueous phase was dialyzed against distilled water and lyophilized to recover the product. The nitrogen content, which varied between 3-6%, was reduced by a single phenol-water extraction to 0.5-0.7% as shown in Table I, column 2. Nitrogen content could be reduced further by sedimenting the endotoxin from 0.5-1.0% aqueous solutions of these preparations at 60,000 × *g* for 4 hours. Data listed in the third column of Table I show that the endotoxin in the sediments contains 0.4-0.6% nitrogen. Small amounts of material containing about 2% nitrogen were removed in the supernatant fluid. The data in the last column of Table I show that about one-half of the remaining nitrogen is accounted for by the hexosamine content of the product as determined by the Elson-Morgan method(1). Thus, there remains very little nitrogenous material to be accounted for. In contrast, it has been our experience that endotoxin isolated directly from *S. enteritidis* cells by the phenol-water procedure contained at least 3% nitrogen even after nucleic acids have been removed.

2. *Treatment of deproteinized aqueous ether extracted endotoxin with lithium aluminum hydride.*

a) *Preparation and chemical and biological properties of the aluminum citrate-endotoxin complex.* The endotoxin used for these experiments was deproteinized by treatment with phenol water as described in the preceding section. Thus, 125 mg of endotoxin which had been dried *in vacuo* over phosphorus pentoxide was suspended in 25 ml of dry dioxane and stirred until a uniform dispersion was obtained. Twenty-five ml of dry ether was added, followed by 125 mg of powdered lithium aluminum hydride. The mixture was refluxed and stirred for 24 hours. The reaction mixture was cooled and treated with 2 ml of 1 M citric acid followed by 20 ml of water and immediately neutralized with 3 N sodium hydroxide. The ether layer was separated and the aqueous layer concentrated *in vacuo* to one-half its volume to remove the dioxane. The residue was diluted to 100 ml and centrifuged at 1550 × *g* for 1 hour to remove a grayish white precipitate. The clear supernatant fluid was dialyzed against distilled water and lyophilized to yield a readily water soluble aluminum citrate-endotoxin complex of high biological potency ("Instant Endotoxin"). Chemical and biological analyses were made by the methods described previously(1,2,3). The pertinent chemical and biological data for several preparations are compared in Table II with those of the

TABLE II. Analysis of $LiAlH_4$-Treated Endotoxin of Low Nitrogen Content from *S. enteritidis*.

	Exp 1		Exp 2		Exp 3		
	Endotoxin	Aluminum citrate complex	Endotoxin	Aluminum citrate complex	Endotoxin	Aluminum citrate complex	Lipid from hydrolysate
Chemical	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Recovery	—	155	—	167	—	170	5
Total fatty acid	4.65	—	7.0	—	5.91	—	31.5
" " " after hydrolysis*	—	2.6	—	1.6	—	1.6	—
Nitrogen	.58	.41	.70	.41	.75	.65	—
Phosphorus	1.10	.62	—	—	1.07	.54	—
Hexose (anthrone)	76	52	63	35.5	64	32	—
Hexosamine	4.0	2.7	3.2	1.85	2.24	1.30	—
Aluminum	.0	5.3	.0	4.0	.0	5.4	—
Citric acid	.0	28.1	—	—	.0	25.1	—
Biological	(μg)	(μg)	(μg)	(μg)	(μg)	(μg)	(μg)
Protection of mice against *S. typhosa* challenge (ED_{50})	.11	.16	.14	.17	.15	.14	>100
Lethality for mice (LD_{50})	350	310	810†	710†	220	270	—
Tumor damage in mice (TD_{50})	.15	.23	.13	.11	.22	.43	>200
Pyrogenicity in rabbits (FI_{40})	.09	.20	.17	.40	.04	.22	>250

* Analysis of lipid from chloroform extract of acid hydrolysate (1 N HCl at 100°C for 45 min).
† Lethality for BCG vaccinated mice(4). Endotoxin: $LD_{50} = 0.025$ μg; Aluminum citrate complex: $LD_{50} = 0.03$ μg.

parent endotoxins; the similarity in composition and potency is evident.

It is especially noteworthy that the aluminum citrate complex was obtained in 155-170% yields based on amount of endotoxin used, although the high biological activity of the starting materials was not significantly affected by this treatment. The reduced proportions of nitrogen, phosphorus, hexose, and hexosamine in the complex accord well with increased yields resulting from incorporation of aluminum and citric acid into the product. Correspondingly, the apparent reduction in total fatty acids is of questionable significance.

The fatty acid analysis of the aluminum citrate complex by the hydroxamic acid method was found to give erratic and unreliable results. This was due to interference by citric acid with the formation of the iron-hydroxamic acid chelate. The aluminum citrate-endotoxin complex was first hydrolyzed in 1 N hydrochloric acid at 100°C for 45 minutes to circumvent this difficulty. The precipitate in the cooled hydrolysate was completely soluble in chloroform and the material recovered by evaporation of the solvent was analyzed for fatty acids in the usual manner. One of the chloroform soluble fractions from an acid hydrolysate (Table II, Exp. 3) was assayed for biological potency and was found to have no detectable endotoxic activity. For bioassay the chloroform solution of the lipoid fraction was shaken with a hot solution of 0.5% Tween 80 in saline and the chloroform was evaporated in a 70°C water bath.

b) *Physical-chemical behavior of the aluminum citrate-endotoxin complex.* The aluminum citrate complex was distinctive because it was instantly soluble in water whereas the phenol treated endotoxin from which it was prepared was dispersed with difficulty. The ultracentrifugation pattern (Fig. 1) of the complex shows the presence of a major boundary having a sedimentation constant of 22.8 Svedberg units ($S = 10^{-13}$ cm/sec/unit field) when examined in a 1% solution in 0.1 M sodium acetate, rotor temperature 20°C. The starting endotoxin, as was usual for phenol treated endotoxin, sedimented so rapidly that it was not possible

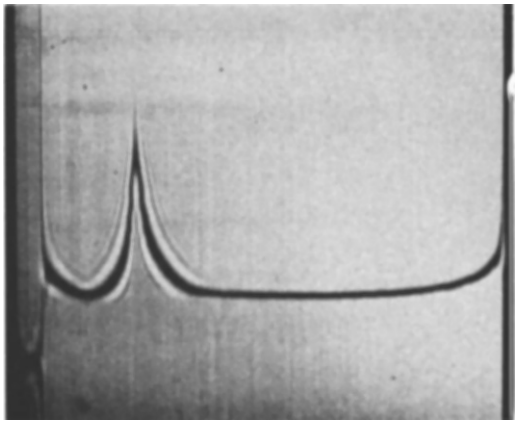

FIG. 1. Ultracentrifugal pattern of aluminum citrate-endotoxin complex of *Salmonella enteritidis*. Examined in 1% solution in 0.1 M sodium acetate 10 min after speed of 50,740 r.p.m. (20°C) was reached. Sedimentation constant = 22.8 S.

to determine its sedimentation constant.

When aluminum citrate was removed from the complex by passing its aqueous solution through a strong acid and base ion exchange column in the salt form, the recovered endotoxin was no longer rapidly soluble in water but its biological potency remained unchanged. At this point it appeared that it might be possible to prepare the complex simply by adding an aluminum citrate solution to an endotoxin solution, dialyzing and lyophilizing to recover the product. However, this was not the case. The procedure led only to recovery of uncombined endotoxin with unchanged solubility.

The haptenic polysaccharide from this endotoxin also reacted with lithium aluminum hydride under the same conditions described to form a similar, but biologically inactive, aluminum citrate complex. In the ultracentrifuge this complex behaved as a single entity with a sedimentation constant of 3.1 S as compared with 1.4 S for the original haptene.

3. *Treatment of conventional aqueous ether extracted endotoxin with lithium aluminum hydride.* An additional experiment included the reaction of lithium aluminum hydride with a conventional aqueous ether extracted endotoxin. This starting material had a considerably higher fatty acid and nitrogen content than the phenol water extracted endotoxin described earlier. Chemical and biological properties of the complex are compared with those of its parent endotoxin in Table III. The fatty acid content has been reduced since incorporated aluminum citrate does not account for reduction of total fatty acid from 10.7% to 1.6%. Fatty acids remaining in the complex were recovered by chloroform extraction of an acid hydrolysate as previously described. This chloroform soluble fraction, amounting to 4% of the endotoxin complex, contained 39.3% fatty acids. The rate of solution of this aluminum citrate complex was not as fast as that of "Instant Endotoxin" despite the greater solubility of the starting material over that of the deproteinized material used for preparation of "Instant Endotoxin."

TABLE III. Analyses of $LiAlH_4$-Treated Conventional Aqueous Ether Extracted Endotoxin from *S. enteritidis*.

	Endotoxin	Aluminum citrate complex
Chemical	(%)	(%)
Recovery	—	107
Total fatty acid	10.7	—
" " " after hydrolysis*	—	1.6†
Nitrogen	3.27	1.38
Phosphorus	.63	.60
Hexose (anthrone)	46	36
Hexosamine	2.2	1.7
Aluminum	.0	5.4
Citric acid	.0	18.2
Biological	(μg)	(μg)
Protection of mice against *S. typhosa* challenge (ED_{50})	.17	.34
Lethality for mice (LD_{50})	220	200
Tumor damage in mice (TD_{50})	.3	.42
Pyrogenicity in rabbits (FI_{40})	.09	.1

* Analysis of lipid from chloroform extract of acid hydrolysate (1 N HCl, 45 min, 100°C).

† Chloroform soluble portion is 4% of the complex containing 39.3% fatty acid.

4. *Treatment of trichloracetic acid extracted endotoxin with lithium aluminum hydride.* Noll and Braude(5) have described the reaction of lithium aluminum hydride with endotoxin extracted by trichloracetic acid (TCA) from *Escherichia coli* (Bovin antigen). They reported the product to be immunogenic but nonpyrogenic and virtually devoid of toxicity. Since products described

TABLE IV. Analyses of $LiAlH_4$-Treated Trichloracetic Acid Extracted Endotoxin from *S. enteritidis*.

	Endotoxin	Aluminum citrate complex	$LiAlH_4$ reduced HCl treated	
			Primary product	Citrate extract
Chemical	(%)	(%)	(%)	(%)
Recovery	—	102	43	57
Total fatty acids	10.8	—	9.6	—
" " " after hydrolysis*	—	2.9	—	—
Nitrogen	2.23	1.03	1.55	1.57
Hexose (anthrone)	53	46	52	33
Hexosamine	2.5	2.4	2.2	1.8
Aluminum	.0	2.4	2.4	6.6
Citric acid	.0	17.6	.0	19.2
Biological	(μg)	(μg)	(μg)	(μg)
Protection of mice against *S. typhosa* challenge (ED_{50})	.35	.52	.4	.6
Tumor damage in mice (TD_{50})	.3	.3	.11	.42
Lethality for mice (LD_{50})	200	310	400	620
Pyrogenicity in rabbits (FI_{40})	.16	.35	.3	.3

* Analysis of lipid from chloroform extract of acid hydrolysate (1 N HCl, 45 min, 100°C).

here retained the biological properties of the parent endotoxin, a TCA extracted endotoxin of *S. enteritidis*(1) was reacted with lithium aluminum hydride. The aluminum citrate complex obtained in 102% yield had the same potency as the starting material. (Table IV, columns 2 and 3). When the reaction mixture was decomposed by addition of 1 mole of hydrochloric acid per mole of lithium aluminum hydride, as described by Noll and Braude, the product was obtained in 43% yield and differed little in biological properties from the parent endotoxin (column 4). The copious aluminum hydroxide precipitate from the reaction mixture was treated with an equimolar amount of citric acid, neutralized with sodium hydroxide and centrifuged. The supernatant fluid was dialyzed and dried from the frozen state to obtain an aluminum citrate-endotoxin complex in 57% yield (column 5) with biological activity only slightly less than that of the primary product. These results indicate that decomposition of the reaction mixture with hydrochloric acid precipitates a considerable amount of endotoxin along with the aluminum hydroxide. In contrast, very little endotoxin is precipitated when the reaction mixture is decomposed with citric acid. When the reaction mixture was decomposed with water alone, also as performed by Noll and Braude, the primary product (51% yield) had lost about two-thirds of its original activity and the aluminum citrate complex extracted from the aluminum hydroxide precipitate (35% yield) was even less active. This loss of activity may have been due to the high alkalinity of the reaction mixture after addition of water.

Discussion. The method described here for reduction of the nitrogen content of aqueous ether extracted endotoxin from *S. enteritidis* by simply treating with phenol water consistently yielded biologically potent products which contained only 0.4-0.5% nitrogen. This is comparable to the lowest value which could be obtained by the previous methods employed which included fractional precipitation with salt and ethanol, according to Webster, followed by phenol water extraction(1). The same result was obtained when the Webster method was replaced by treatment with the enzyme pronase. The 0.4% nitrogen content in aqueous ether extracted endotoxin which had been treated directly with phenol could not be reduced further by the action of pronase.

Inasmuch as protein-free endotoxin preparations have been reported to contain 1-2% nitrogen(6), it appears that the method de-

scribed here removes not only protein but also other nitrogenous materials such as peptide-like substances. This pronounced reduction in nitrogen content has been effected only with endotoxins prepared by the aqueous ether procedure. It follows, therefore, that the phenol-water method extracts from whole cells nitrogenous materials which are not an integral part of the endotoxin and which are not extracted by the aqueous ether procedure; conversely, the aqueous ether method extracts nitrogenous material from whole bacteria which can subsequently be removed by the phenol-water procedure.

The treatment with lithium aluminum hydride of endotoxin prepared by extraction with aqueous ether or with TCA resulted in removal of about one-half of the firmly bound fatty acids in these preparations. Treatment of deproteinized aqueous ether extracted endotoxin, however, did not result in a further significant reduction of fatty acid ester content, but the endotoxin was modified in such a manner as to allow formation of an aluminum citrate complex of high biological activity. Because this complex is rapidly soluble in water, the designation "Instant Endotoxin" was chosen for it. In contrast to most conventional endotoxins, this product gives clear or only slightly opalescent solutions at 1% concentration. This behavior may be attributed to the ability of aluminum citrate to form addition compounds or oxonium salts with alcoholic hydroxyl groups, which have a greater affinity for protons than aqueous hydroxyl groups. A known consequence of this phenomenon is the increased solubility in water of substances such as polysaccharides which are otherwise practically insoluble in this menstruum(7). This explanation is further substantiated by the behavior of the endotoxin recovered from the aluminum citrate complex by passage of its aqueous solution through a mixed-bed ion exchange column. The endotoxin, which is then free of citrate and contains only traces of aluminum, is no longer readily soluble.

The content of fatty acid ester and amide in the aluminum citrate-endotoxin complex is about 2%, expressed as palmitic acid and determined by the hydroxamic acid method (3). Fatty acid-containing material which could be liberated as a completely chloroform soluble precipitate by hydrochloric acid hydrolysis of the complex lacked host reactive properties; it exerted even less activity than preparations designated as lipid A(2).

Noll and Braude(5) recently reported the preparation of a nonpyrogenic, virtually nontoxic, but highly immunogenic fraction, by treatment of TCA extracts from *E. coli* with lithium aluminum hydride. To isolate this fraction, they decomposed the reaction mixture with either water or hydrochloric acid and filtered to obtain a sediment consisting of potent endotoxin co-precipitated with aluminum hydroxide and a supernatant fluid containing a nontoxic but immunogenic fraction in about 10% yield. These authors stated that both the biologically intact endotoxin and the portion whose toxicity and pyrogenicity had been selectively reduced were free of ester-bound fatty acids.

When endotoxin from *S. enteritidis* isolated by either the aqueous ether or the TCA method was treated with lithium aluminum hydride and the reaction mixture decomposed by the method of Noll and Braude, approximately one-half of the endotoxin was precipitated with the aluminum hydroxide. The material recovered from the supernatant fluid retained pyrogenicity, toxicity and the ability to stimulate nonspecific resistance of mice to challenge with *Salmonella typhosa*. It also retained up to 2% fatty acids. The endotoxin separated from the aluminum hydroxide precipitate by extraction with citric acid solution also retained these biological properties.

Noll and Braude did not report chemical analyses of their materials. They correlated the loss of pyrogenicity and toxicity with the disappearance of the ester absorption bands in the infrared spectra. In the present work, the change in the infrared spectra of lithium aluminum hydride treated *S. enteritidis* endotoxin was similar to that described by Noll and Braude (disappearance of the 5.8 $m\mu$ absorption band). However, we also found that treatment of deproteinized aqueous ether extracted endotoxin with lithium aluminum hydride resulted in the same changes

in the infrared spectrum despite little or no removal of fatty acid esters from the substance. These results together with those reported by Noll and Braude make it clear that no evidence has been established for correlation between fatty acid ester content and toxic properties of the endotoxin. It is conceivable that, in the case of *E. coli,* the endotoxin in the Boivin extract is accompanied by nontoxic protective substances. The treatment with lithium aluminum hydride then would not selectively modify a portion of the endotoxin and render it nontoxic, but would separate the nontoxic antigen from the endotoxin by subsequent precipitation with aluminum hydroxide. Further discussion is best deferred until the nontoxic antigenic fraction from *E. coli* has been chemically characterized.

Summary. Treatment of conventional aqueous ether extracted endotoxins from *Salmonella enteritidis* with aqueous phenol, followed by high speed centrifugation, yielded material having high biological potency with a nitrogen content of 0.4-0.5% and a fatty acid content of 3-4%. Upon reacting this purified endotoxin with lithium aluminum hydride and citric acid solution, an aluminum citrate-endotoxin complex was recovered which had the desirable features of high biological potency and a rapid rate of solution in water. Non-phenolized endotoxins extracted from *S. enteritidis* with aqueous ether or trichloroacetic acid also formed aluminum citrate complexes under the same conditions. These were biologically potent but somewhat more difficult to dissolve. After prolonged treatment with lithium aluminum hydride, the aluminum citrate complexes still contained 1-2% esterified fatty acids. Lipid fractions isolated from acid hydrolysates of the complexes were biologically inactive.

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Effect of Hydrocortisone on Experimental Silicotic Nodule.* (27967)

ROBERT C. TALLEY AND BENJAMIN BURROWS (Introduced by Stephen Rothman)
Department of Medicine, University of Chicago, Chicago, Ill.

There have been conflicting reports on the effects of corticosteroids on the progression and maturation of the silicotic nodule, one report even denying that steroids reduce total fibrosis(1). While a number of other studies have confirmed a diminished quantity of fibrosis in steroid-treated animals(2-9), some have suggested that this is due to an arrest in the transformation of reticulin to collagen(7), while others indicate that the maturity of fibrosis is unaffected by corticosteroid administration(4,5). Both decreased cellularity of treated lesions(2,3) and increased cellularity(7) have been reported. In the opinion of some investigators, the principal effect of cortisone is on the migration of dust cells and dispersion of silica dust(1,4,6).

The present study concerns the effect of hydrocortisone on the progression and maturation of the silicotic nodule. In an attempt to minimize the effects of steroids on the mo-

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