DISSOCIATION OF THE BIOLOGICAL PROPERTIES OF BACTERIAL ENDOTOXIN BY CHEMICAL MODIFICATION OF THE MOLECULE

By HENRY H. FREEDMAN, Ph.D., and BARNET M. SULTZER, Ph.D.

(From the Princeton Laboratories, Inc., Princeton)

(Received for publication, June 6, 1962)

Downloaded from http://rupress.org/jem/article-pdf/116/6/929/1080888/929.pdf by guest on 09 August 2022

Interest in the bacterial endotoxins arises, not only from their specific antigenicity and from the phenomenon of tolerance, but, equally, from the remarkable ability of these pyrogenic and highly toxic complexes to stimulate, at microgram doses, substantial refractoriness to a variety of ordinarily lethal insults.

Endotoxin treatment has been shown to protect against the consequences of infection with Gram-negative, Gram-positive, or acid-fast bacteria (1–4), and to promote resistance to the lethality of hemorrhagic or traumatic shock (5–7) or whole-body x-irradiation (8, 9). Despite their wide spectrum of activities (10, 11), no unequivocal evidence for experimental dissociation of the *in vivo* non-specific properties of endotoxins by chemical modification of the molecule has been reported.

The presence of lipid, carbohydrate, and, usually, protein moieties within the endotoxin molecule has encouraged hydrolytic approaches designed to recover one or another of these components. Hydrolysis with acid or base leads to destruction of all *in vivo* activities, but, where active material has been recovered following hydrolysis, the whole range of properties, toxic and protective, has been present. This is perhaps best illustrated by the current difference of opinion on the role of the lipid component (12, 13). To avoid hydrolytic or oxidative alterations, studies on the chemical modification of endotoxin were undertaken using reactions resulting in substitution at functional groups known to be present in the molecule. This and the following paper (14) describe the acylation of endotoxin, with recovery of a fraction exhibiting in the gross reduced pyrogenicity and lethality but retaining the ability to stimulate so-called non-specific host resistance to infection. A preliminary report of our findings has been published (15). While our manuscripts were in preparation, Noll and Braude (16) reported the detoxification of endotoxin by reductive cleavage of ester bonds yielding a preparation of high immunogenic potency, which, unlike our preparation, confers tolerance to the parent endotoxin.

Materials and Methods

Chemical.—Several endotoxin preparations have been used as starting materials for a variety of substitution reactions. This and the following report (14) describe results obtained with acetylation of a Boivin-type endotoxin derived from *Salmonella typhosa* O-901 (Difco

Laboratories, Inc., Detroit). Nitrogen content, by micro-Kjeldahl digestion with selenium-sulfuric acid and nesslerization, was, for a number of individual batches, close to 5 per cent. In all preparative procedures precautions to avoid contamination by extraneous pyrogens were rigorously observed. All glassware and other apparatus were baked at 175–180°C for 2 to 3 hours before use and saline and distilled water were proven non-pyrogenic. The preparative procedure has been briefly described (15); a detailed description of a larger run follows. To 800 mg of endotoxin were added 150 ml of acetic anhydride and 80 mg of anhydrous powdered sodium acetate as basic catalyst. The reaction mixture, in a capped centrifuge bottle, was heated in a boiling water bath for 2 hours with occasional mixing. After cooling, the acetic anhydride was removed as a clear solution by centrifugation. The insoluble material remaining was freed of anhydride by drying at room temperature under a stream of nitrogen, and was then ground to a fine powder. This was washed 3 times with 100 ml portions of saline and finally with water. The washes were removed by centrifugation at low speed and were combined, yielding an opalescent suspension. The washed precipitate was dried under N_2 and ground to a light tan powder, designated fraction P, amounting to 40 to 45 per cent of the starting material. The yields of P for runs of 100, 600, and 800 mg were 45, 260, and 320 mg, respectively. The combined washes were dialyzed against 3 changes of 20 volumes of pyrogen-free water, then freeze-dried, with recovery of 20 to 25 per cent of the starting material, this fraction being designated E. The acetic anhydride solution from small runs was evaporated to dryness under N_2 with gentle heating (30°C); with larger quantities, solids were recovered by pouring the anhydride solution into excess cold water and filtering. This anhydride-soluble, water-insoluble fraction was soluble in chloroform in which it was transferred to a vial for evaporation and weighing; a yield of 30 to 35 per cent was obtained from direct evaporation, designated fraction L.

Fraction P, which is the principal subject of this report, is strongly lyophobic. A stock solution at 1 mg/ml may be readily prepared by adding approximately $\frac{1}{10}$ the final volume of saline and triturating with a spatula, taking care to thoroughly wet and disperse the solids. Additional saline is then added and any undispersed aggregates are worked into suspension. Saline to the final volume may then be added. The saline suspension is denser than that of the endotoxin at the same concentration and settles more readily, requiring thorough mixing before sampling. Fraction E behaves exactly like the original endotoxin. The chloroform-soluble fraction L has been dispersed by dissolving in a small amount of $CHCl_3$, adding saline to the desired volume, then blowing out the $CHCl_3$ with a fine stream of N_2. In some experiments, described below, tween 80 was added before removing the $CHCl_3$ to improve the saline dispersion.

Biological.—Testing for pyrogenicity was done in male rabbits of mixed breed weighing 2 to 2.5 kg. Saline dilutions were injected *via* a marginal ear vein. The preparation of animals for, and handling during, testing were as previously described (17). Lethality was determined in ICR male mice weighing 18 to 20 gm with deaths recorded for 72 hours. Carbon clearance was measured in mice by the method of Biozzi, *et al.* (18) using the Gunther Wagner C11/1431a carbon suspension, as previously described (19).

In all instances groups of animals receiving endotoxin or modified fractions were tested at the same time. Each derivative was tested against its parent endotoxin for all parameters studied. The procedures for individual experiments are described below.

EXPERIMENTAL

Acetyl content, determined by saponification with 0.02 N NaOH at 55°C for 30 minutes and titration with 0.01 N HCl to phenol red end-point, was 1.5 to 2 per cent for the endotoxin, 8 to 12 per cent for fraction P, 3 to 3.5 per cent for fraction E, and 7 to 8 per cent for fraction L, the range representing a number

Downloaded from http://rupress.org/jem/article-pdf/116/6/929/1080888/929.pdf by guest on 09 August 2022

of individual preparations. It should be noted that the consumption of base by the endotoxin is equivalent to, but not necessarily attributable to, the stated acetyl content. The pertinence of the alkaline-labile acetyl groups is supported by the recovery of the original pyrogenicity and lethality following saponification of fraction P (*vide infra*), suggesting strongly that O-acetylation accounts for the observed dissociations of biological activity.

Pyrogenicity.—The marked reduction in pyrogenicity exhibited by fraction P is shown in Figs. 1 and 2, which represent derivatives from two different endo-

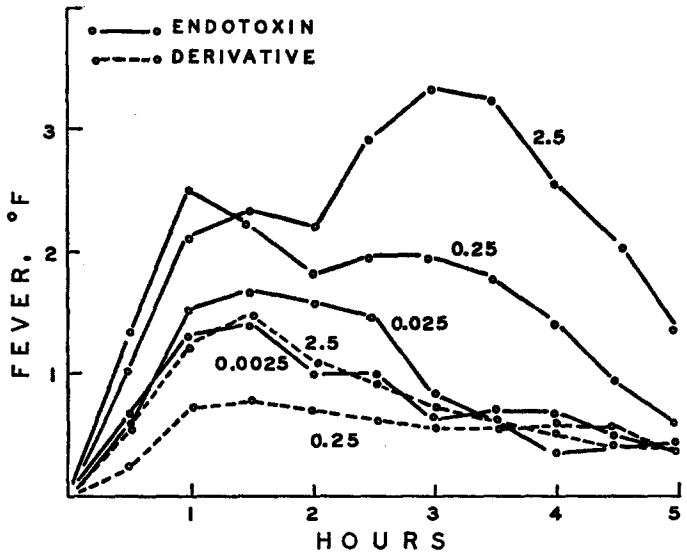

FIG. 1. Mean febrile responses produced by parent endotoxin and its acetylated fraction P, in groups of 5 to 20 rabbits given indicated doses (in micrograms).

toxin preparations. A total of 57 rabbits was distributed among the doses for the experiments described in Fig. 1, and 73 rabbits for those in Fig. 2. The data indicate reductions in pyrogenicity of 1000-fold and 100-fold, respectively. These experiments represent the upper and lower limits of decrease in pyrogenicity for all preparations covered by this and the following paper (14). The retention of original pyrogenicity in fraction E, and the non-pyrogenicity of fraction L, are described in Fig. 3. Dispersing the L fraction with the aid of 0.05 per cent tween 80 prior to diluting did not alter its non-pyrogenicity.

Lethality.—In Fig. 4 are plotted the data from experiments comparing the lethality by intraperitoneal route of 5 individual P fractions with that of the parent endotoxin. A total of 167 mice[1] received doses of the endotoxin and 208

[1] As have others (20), we have been unsuccessful in attempting to establish an LD_{50} for endotoxin in the rabbit. In one experiment, using a dose of 2 mg, iv, 3 of 4 rabbits given the parent endotoxin died whereas all 4 treated with fraction P survived.

Downloaded from http://rupress.org/jem/article-pdf/116/6/929/1080888/929.pdf by guest on 09 August 2022

mice were used for the five derivatives. The LD_{50} for the endotoxin is approximately 0.25 mg, whereas for the more toxic of the derivatives this value is about 2 mg. Endotoxin is somewhat more effective when given by intravenous, rather than intraperitoneal, route, the LD_{50} falling by as much as one-half (21). The opposite was found for fraction P which proved less lethal when administered intravenously; this is described in Table I. At the 5 mg dose all mice injected ip were dead within 16 hours and the single death in the iv group oc-

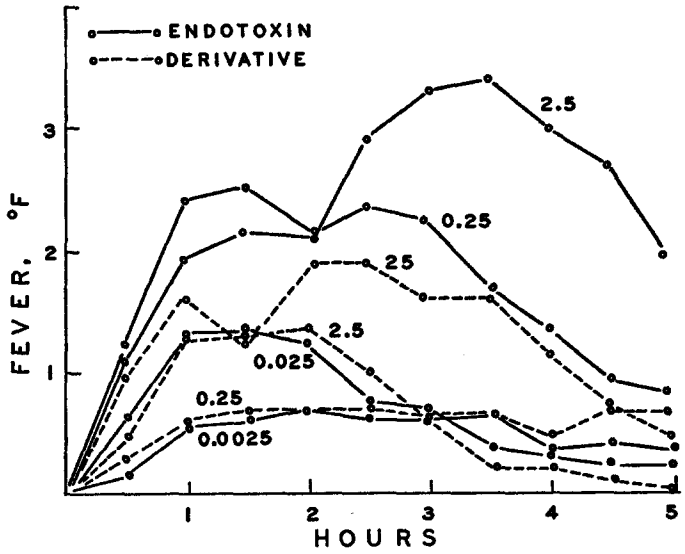

FIG. 2. Mean febrile responses produced by parent endotoxin and its acetylated fraction P, in groups of 8 to 25 rabbits given indicated doses (in micrograms).

curred at 30 hours. A comparison of the lethality of the parent endotoxin when given by ip and iv routes is included in Table I; an approximately 2-fold increase in potency by the latter route is evident. It is obvious that estimates of degree of reduction of toxicity by the two routes of administration would yield very different values.

The retention of original lethality in fraction E and its absence in fraction L are shown in Table II. Since the chloroform-soluble L fraction might appear nonlethal because of the difficulty in dispersing the material in saline, an experiment was done in which tween 80 was added before removing the $CHCl_3$. This procedure resulted in a fine dispersion of L in saline, but did not alter its lack of lethality compared to the endotoxin containing the same concentration of tween.

Reversal of Detoxification by Deacetylation.—Recovery of the original lethality

Downloaded from http://rupress.org/jem/article-pdf/116/6/929/1080888/929.pdf by guest on 09 August 2022

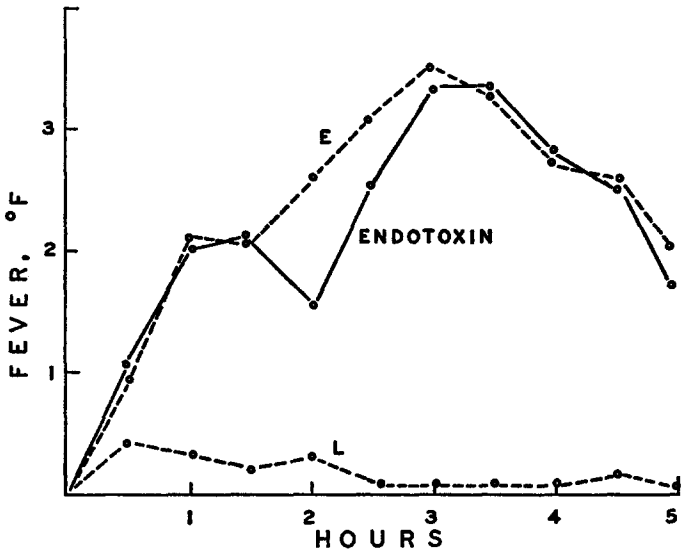

FIG. 3. Comparison of pyrogenicity of fractions L and E with that of parent endotoxin at 2.5 μg doses in groups of 4 to 6 rabbits.

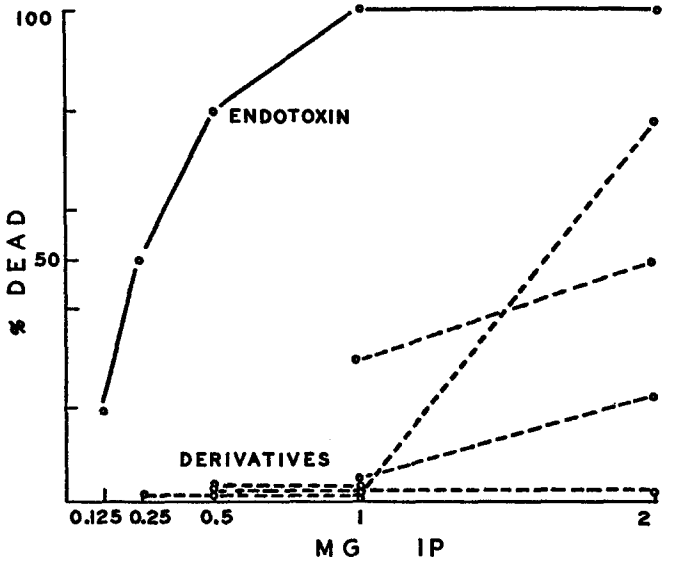

FIG. 4. Lethality for mice of parent endotoxin and 5 individual P fractions, given ip.

and pyrogenicity by mild saponification of fraction P was demonstrated as follows:

Both endotoxin and fraction P, at 2 mg/ml in saline, were brought to 0.01 N base by addition of N NaOH. Aliquots of each were withdrawn immediately and neutralized with N

Downloaded from http://rupress.org/jem/article-pdf/116/6/929/1080888/929.pdf by guest on 09 August 2022

HCl, these serving as control preparations. The remaining portions of each were heated in a water bath at 55°C for 30 minutes, then neutralized with N HCl. Groups of mice were given 0.5 ml doses ip for determination of lethality. For pyrogen studies the same system was used, except that the concentrations of endotoxin and derivative were 1 or 10 μg/ml and doses of 0.25 ml were given rabbits iv. In initial experiments equivalent pyrogenic doses of the endotoxin and fraction P were saponified, and the endotoxin at such high dilution (0.01 μg/ml) suffered a loss of activity. Both materials at 2 mg/ml cleared on saponification.

TABLE I

Comparison of Lethality for Mice of Parent Endotoxin and Fraction P by Intraperitoneal and Intravenous Routes of Administration

Group	Dose	Dead/total	
		ip	iv
	*mg**		
Endotoxin	0.05	0/10	2/10
"	0.10	0/15	13/35
"	0.20	3/10	6/10
Fraction P	3	4/5	1/5
"	5	5/5	1/4

* All preparations given in 0.2 ml saline.

TABLE II

Lethality for Mice of Acetylated Fractions E and L Injected Intraperitoneally

Group	Dead/total Dose, mg	
	0.3	0.6
Endotoxin	4/10	9/10, 7/7*
Fraction E	6/10	10/10
" L	—	0/10, 0/5*

* In 0.05 per cent tween 80.

In Table III are given the results of two experiments demonstrating the recovery of original lethality in fraction P following deacetylation. The recovery of pyrogenicity is shown in Fig. 5.

Failure to Confer Tolerance to Parent Endotoxin.—For investigation of the well known refractoriness to a lethal dose of endotoxin which follows a prior sublethal dose, the following experimental model was used.

Groups of mice were given the parent endotoxin or fraction P at 50 μg in 0.5 ml of saline, ip, and a control group was given the saline only. Two or 3 days later all animals were challenged with an estimated LD_{75} dose (0.6 mg, ip) of the parent endotoxin.

Downloaded from http://rupress.org/jem/article-pdf/116/6/929/1080888/929.pdf by guest on 09 August 2022

The data in Table IV make clear that the derivative does not confer tolerance to the lethality of the parent endotoxin. Since this finding might reflect only a loss of specific immunizing potency, and since the tolerance following a sublethal dose of endotoxin extends to heterologous endotoxins, an experiment was

TABLE III

Recovery of Original Toxicity in Fraction P by Deacetylation

Group*	Dead/total
Endotoxin, control	13/20
" saponified	13/20
Fraction P, control	0/20
" saponified	14/20

* Each group received 0.5 ml, ip, of 2 mg/ml preparations; see text for details.

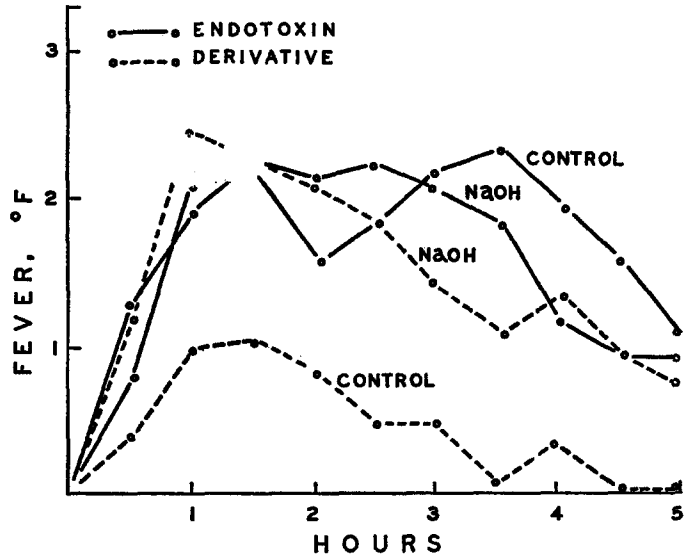

FIG. 5. Recovery of pyrogenicity in fraction P following saponification. Groups of 4 rabbits each given 0.25 μg doses of control or NaOH-treated preparations.

done in which the challenge was 0.4 mg, ip, of the heterologous *Escherichia coli* 0127:B8 endotoxin (Difco Laboratories, Inc.). The groups pretreated with saline or with 20 μg of the derivative of the *S. typhosa* endotoxin 2 days earlier suffered 40 and 50 per cent deaths, respectively, whereas the group given 20 μg of the parent *S. typhosa* endotoxin were protected, 20 per cent dying.

A variety of experimental approaches was employed to study tolerance to the pyrogenicity of endotoxin. In one set of experiments groups of 3 or 4 rabbits each were given equal doses

Downloaded from http://rupress.org/jem/article-pdf/116/6/929/1080888/929.pdf by guest on 09 August 2022

of parent endotoxin or fraction P (0.25 or 2.5 μg) on the 1st day, the febrile responses were recorded, then, the following day, all animals were treated with the same dose of either the parent endotoxin or the heterologous *E. coli* endotoxin. Alternatively, groups of rabbits were given estimated equally pyrogenic doses of endotoxin or derivative, *e.g.* 0.025 and 2.5 μg, respectively, and the next day were tested with the parent endotoxin at its original, or at an increased (intermediate), dose.

TABLE IV

Failure of Fraction P to Confer Tolerance to Lethality of Parent Endotoxin

Treatment* Day 1	Challenge* Day 4	Dead/total
Saline	0.6 mg Endotoxin	16/20
Endotoxin, 50 μg	" " "	5/19
Fraction P, 50 μg	" " "	13/20

* All preparations administered ip.

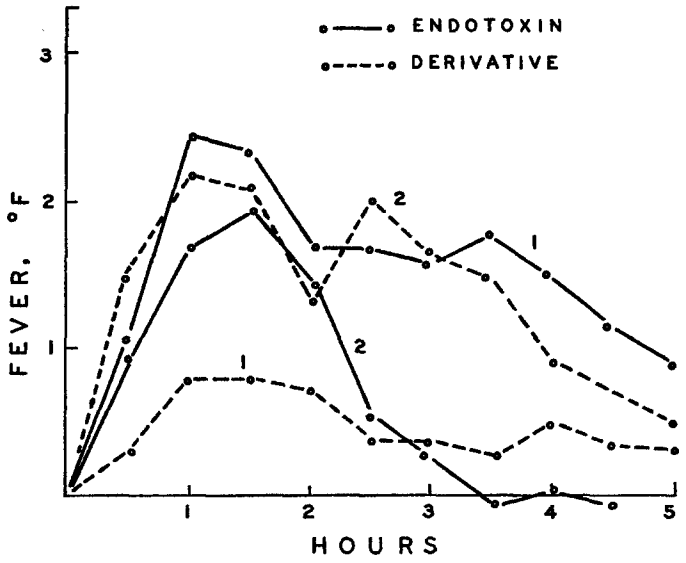

FIG. 6. Mean febrile responses of groups of 4 rabbits each given 0.25 μg of parent endotoxin or fraction P on day 1 and challenged with 0.25 μg endotoxin on day 2.

The results of a representative experiment, in which 4 rabbits each were given 0.25 μg of endotoxin or its derivative on day 1 and tested with the same dose of the homologous endotoxin on day 2, are given in Fig. 6. The derivative at an equal dose has clearly not retained the ability to confer tolerance to the pyrogenicity of the endotoxin. When the heterologous *E. coli* endotoxin was used as

Downloaded from http://rupress.org/jem/article-pdf/116/6/929/1080888/929.pdf by guest on 09 August 2022

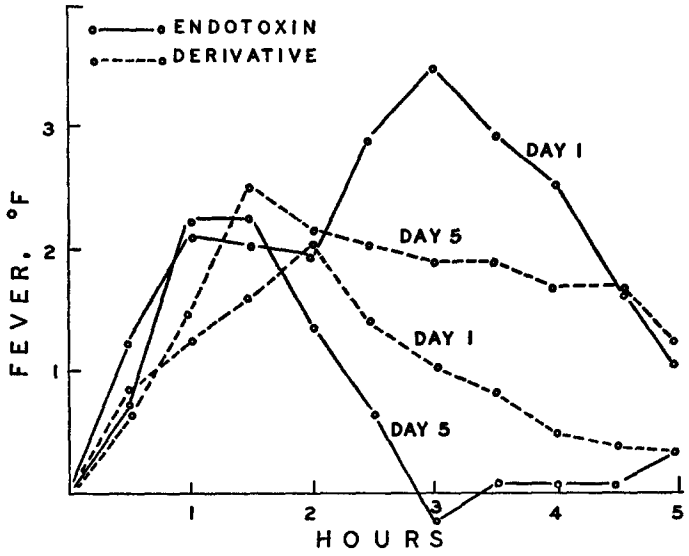

FIG. 7. Mean febrile responses on days 1 and 5 of groups of 3 rabbits each given parent endotoxin or fraction P daily on stepped dose schedule.

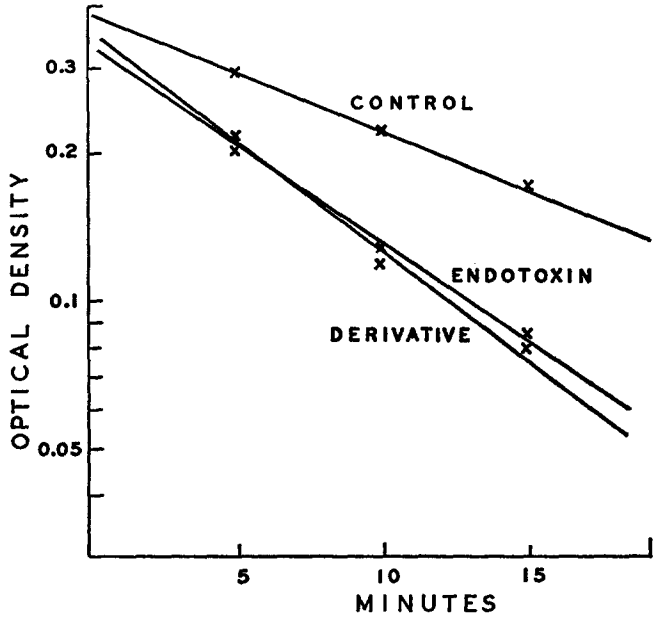

FIG. 8. Rates of clearance of carbon particles from the blood, at a dose of 12 mg/100 gm body weight, in groups of 6 mice each, given 10 μg of parent endotoxin or its fraction P, ip, 24 hours earlier.

Downloaded from http://rupress.org/jem/article-pdf/116/6/929/1080888/929.pdf by guest on 09 August 2022

the test pyrogen on day 2, the same result was obtained. From experiments with estimated equally pyrogenic doses of parent endotoxin and fraction P it appears that the degree of tolerance to endotoxin induced by the derivative is a function of the pyrogenicity of the preparatory dose selected.

It was of interest to determine whether the derivative would confer tolerance to its own pyrogenicity. Two groups of rabbits were given daily injections of endotoxin or derivative according to the following schedule: days 1 and 2, 2.5 μg; days 3 and 4, 5.0 μg; days 5 and 6, 10.0 μg. The group treated with fraction P showed definite tolerance to the pyrogenicity of the second equal dose on day 2, but it failed to parallel the endotoxin-treated group in developing refractoriness to the pyrogenicity of increasing dose; this is shown in Fig. 7, in which the febrile responses of the two groups are compared for days 1 and 5.

Stimulation of Phagocytic Activity.—In Fig. 8 the enhancement of rate of clearance of carbon particles from the blood 24 hours after doses of 10 μg of either parent endotoxin or fraction P, given ip, is shown. Although the derivative clearly retains this stimulatory activity in the experimental model described, further studies have revealed differences in the time-course of alteration of phagocytic activity, markedly route-dependent, which will be described separately (22).

DISCUSSION

At the time the initial findings described above were made we were aware of Goebel's work on modification of *Shigella* endotoxin, in which acetylation with ketene did not alter toxicity (23). Subsequently, our attention was directed to the work of Treffers (24) on the soluble somatic antigen of *Shigella*, prepared by the diethylene glycol method, acetylation of which in aqueous pyridine resulted in decrease of acute toxicity and pyrogenicity with retention of specific immunizing activity against intracerebral challenge with live *Shigella* organisms in the mouse. The acetylation in pyridine of lipopolysaccharide endotoxin, to yield a chloroform-soluble, detoxified derivative has also been described by Westphal and associates (25). Our own water-insoluble, chloroform-soluble fraction L, and the chloroform-soluble derivative we have obtained from acetylation in aqueous pyridine, have both been thoroughly detoxified but are incapable of stimulating non-specific host resistance to infection (14).

The three fractions isolated after acetylation of endotoxin by the method described range in properties from retention of full endotoxic potency for fraction E to complete inactivity for fraction L. Fraction P is of primary interest for it has allowed the experimental dissociation of some of the myriad biological effects of the parent endotoxin. Since the original toxicity and pyrogenicity are recovered in P by deacetylation, there is good reason to attribute the observed separation of biological properties to the acetyl groups introduced. The larger question, whether

Downloaded from http://rupress.org/jem/article-pdf/116/6/929/1080888/929.pdf by guest on 09 August 2022

the altered biological activities reflect substitution at particular reactive sites or, rather, follow from consequent physical changes in the molecule as a whole, cannot, even though the reaction is reversible, be answered with any degree of certainty. That the derivative may undergo deacetylation *in vivo*, with release of the then active endotoxin at some unknown rate, immediately suggests itself. This appears extremely unlikely when one contrasts the retention of non-specific immunizing effect with the failure to confer tolerance to endotoxin at equivalent doses and time intervals. The pretreated animals cannot anticipate the subsequent challenge, and if protection is assumed to result from deacetylation *in vivo* in the one case, the reversion must also have occurred in the other. This argument is independent of possible differences in the mechanisms by which the two responses are expressed. The altered physical state, at least as revealed by the gross difference in appearance and stability of saline dispersions, appears equally unlikely to explain the differences in biological response. Separation of endotoxin preparations into fractions of widely varying particle size, *e.g.* by density gradient centrifugation, has not revealed differences in biological activity (26, 27), and it is well known that highly insoluble endotoxins easily removed from aqueous dispersion at low centrifugal speeds may be fully as pyrogenic as those produced by methods yielding relatively "soluble" products (28). We (29) have been able to alter the parent endotoxin so that extremely coarse, rapidly settling dispersions in saline are obtained, with no loss of pyrogenicity.

Of particular interest is the failure of the P fraction to provide equivalent tolerance to the lethality or pyrogenicity of the homologous or an heterologous endotoxin, while retaining the ability both to promote non-specific resistance to infection (14) and to stimulate phagocytic activity of the reticuloendothelial system. The tolerance to endotoxic effects that develops after initial exposure has been explained on the basis of increased phagocytic activity of the RES. It must be noted, however, that we have not measured the clearance of endotoxin itself, and it has been demonstrated that an experimental procedure that results in depression of clearance for one colloid may increase the rate of clearance of another (30). Further, it has recently been suggested that the stimulatory effect upon phagocytosis produced by serum from endotoxin-treated animals (19) is attributable to increased opsonic activity of such serum and that there may be a variety of such opsonins (31).

Studies on fraction P and its behavior in other systems in which endotoxins are active are continuing.

SUMMARY

The preparation and properties of three fractions isolated from the acetylation of endotoxin by acetic anhydride in the presence of anhydrous sodium acetate are described. One fraction is of primary interest for it allows the experimental

Downloaded from http://rupress.org/jem/article-pdf/116/6/929/1080888/929.pdf by guest on 09 August 2022

dissociation of some of the myriad biological activities of the parent endotoxin. Pyrogenicity and acute toxicity are markedly reduced, and ability to confer tolerance to the lethality and pyrogenicity of homologous or heterologous endotoxins is equivalently diminished. In contrast, this acetylated derivative retains the ability to stimulate phagocytosis and, as shown in the following paper, to enhance non-specific host resistance to infection. Detoxification is reversible, the original toxicity and pyrogenicity being recovered following mild saponification.

The implications of the findings are discussed, and it is suggested that the observed dissociation of biological properties is explained neither by deacetylation of the derivative *in vivo* nor by the observable alteration of physical state in the gross.

We are indebted to Professor A. S. Gordon for suggesting we undertake studies on chemical modification of endotoxin, and for his encouragement and criticism.

The skilled technical assistance of Miriam Graff and Armin Brahm, and assistance in performance of pyrogen assays by Romus Broadway, are gratefully acknowledged.

BIBLIOGRAPHY

1. Rowley, D., Rapidly induced changes in the level of nonspecific immunity in laboratory animals, *Brit. J. Exp. Path.*, 1956, **37,** 223.
2. Landy, M., and Pillemer, L., Increased resistance to infection and accompanying alteration in properdin levels following administration of bacterial lipopolysaccharides, *J. Exp. Med.*, 1956, **104,** 383.
3. Dubos, R. J., and Shaedler, R. W., Reversible changes in the susceptibility of mice to bacterial infections. I. Changes brought about by injection of pertussis vaccine and bacterial endotoxins, *J. Exp. Med.*, 1956, **104,** 53.
4. Boehme, D., and Dubos, R. J., The effect of bacterial constitutents on the resistance of mice to heterologous infections and on the activity of their reticuloendothelial system, *J. Exp. Med.*, 1958, **107,** 523.
5. Zweifach, B. W., and Thomas, L., The relationship between the vascular manifestations of shock produced by endotoxin, trauma, and hemorrhage. I. Certain similarities between the reactions in normal and endotoxin-tolerant rats, *J. Exp. Med.*, 1957, **106,** 385.
6. Smiddy, F. G., and Fine, J., Host resistance to hemorrhagic shock. X. Induction of resistance by shock plasma and by endotoxins, *Proc. Soc. Exp. Biol. and Med.*, 1957, **96,** 558.
7. Fine, J., Frank, E. D., Ravin, H. A., Rutenberg, S. H., and Schweinburg, F. B., The bacterial factor in traumatic shock, *New England J. Med.*, 1959, **260,** 214.
8. Smith, W. W., Alderman, I. M., and Gillespie, R. E., Increased survival in irradiated animals treated with bacterial endotoxins, *Am. J. Physiol.*, 1957, **191,** 124.
9. Zweifach, B. W., Kivy-Rosenberg, E., and Nagler, A. L., Resistance to whole-body X-irradiation in rats made tolerant to bacterial endotoxins, *Am. J. Physiol.*, 1959, **197,** 1364.

Downloaded from http://rupress.org/jem/article-pdf/116/6/929/1080888/929.pdf by guest on 09 August 2022

10. Thomas, L., The physiological disturbances produced by endotoxins, *Ann. Rev. Physiol.*, 1954, **16,** 467.
11. Bennett, I. L., Jr., and Cluff, L. E., Bacterial pyrogens, *Pharmacol. Rev.*, 1957, **9,** 427.
12. Mihich, E., Westphal, O., Luderitz, O., and Neter, E., The tumor necrotizing effect of lipoid A component of *Escherichia coli* endotoxin, *Proc. Soc. Exp. Biol. and Med.*, 1961, **107,** 816.
13. Ribi, E., Haskins, W. T., Landy, M., and Milner, K. C., Symposium on bacterial endotoxins. I. Relationship of chemical composition to biological activity, *Bact. Rev.*, 1961, **25,** 427.
14. Sultzer, B. M., and Freedman, H. H., The stimulation of non-specific host resistance to infection by chemically modified endotoxin, *J. Exp. Med.*, 1962, **116,** 943.
15. Freedman, H. H., Sultzer, B. M., and Kleinberg, W., Detoxification of bacterial endotoxin with retention of ability to stimulate non-specific resistance to infection, *Proc. Soc. Exp. Biol. and Med.*, 1961, **107,** 819.
16. Noll, H., and Braude, A. I., Preparation and biological properties of a chemically modified *Escherichia coli* endotoxin of high immunogenic potency and low toxicity, *J. Clin. Inv.*, 1961, **40,** 1935.
17. Freedman, H. H., Passive transfer of tolerance to pyrogenicity of bacterial endotoxin, *J. Exp. Med.*, 1960, **111,** 453.
18. Biozzi, G., Benacerraf, B., and Halpern, B. N., Quantitative study of granulopectic activity of the reticulo-endothelial system. II. A study of the kinetics of the granulopectic activity of the R.E.S. in relation to the dose of carbon injected, *Brit. J. Exp. Path.*, 1953, **34,** 441.
19. Freedman, H. H., Reticuloendothelial system and passive transfer of endotoxin tolerance, *Ann. New York Acad. Sc.*, 1960, **88,** 99.
20. Thomas, L., Pyrogens, *in* Polysaccharides in Biology, (G. F. Springer, editor) New York, Josiah Macy, Jr. Foundation, 1957, 194.
21. Noyes, H. E., McInturf, C. R., and Blahuta, G. J., Studies on distribution of *Escherichia coli* endotoxin in mice, *Proc. Soc. Exp. Biol. and Med.*, 1959, **100,** 65.
22. Freedman, H. H., and Sultzer, B. M., Alterations of phagocytic activity in relation to non-specific immunity, *in* Role of the RES in Immunity, International Symposium, (B. N. Halpern, editor), Centre National de la Recherche Scientifique, Gif-sur-Yvette, in press.
23. Goebel, W. F., Binkley, F., and Perlman, E., Studies on the Flexner group of dysentery bacilli. I. The specific antigens of *Shigella paradysenteriae* (Flexner), *J. Exp. Med.*, 1945, **81,** 315.
24. Treffers, H. P., The detoxification by acetylation of soluble antigens from *Shigella dysenteriae* (Shiga) and *E. typhosa*, *Science*, 1946, **103,** 387.
25. Keiderling, W., Wohler, F., and Westphal, O., Uber bakterielle Reizstoffe. IV. Experimentelle Untersuchungen zur Differenzierung der therapeutischen Wirkungen von bakterieller Vaccine, reinem Polysaccharid-Pyrogen und acetylierten Polysaccharid-Derivaten aus gramnegativen Bakterien, *Arch. exp. Path. u. Pharmakol.*, 1953, **217,** 293.

Downloaded from http://rupress.org/jem/article-pdf/116/6/929/1080888/929.pdf by guest on 09 August 2022

26. Roberson, B. S., and Schwab, J. H., Endotoxic properties associated with cell walls of Group A streptococci, *J. Infect. Dis.*, 1961, **108,** 25.
27. Webster, M. E., Sagin, J. F., Landy, M., and Johnson, A. G., Studies on the O antigen of *Salmonella typhosa*. I. Purification of the antigen, *J. Immunol.*, 1955, **74,** 455.
28. Westphal, O., Pyrogens, *in* Polysaccharides in Biology (G. F. Springer, editor), New York, Josiah Macy, Jr. Foundation, 1957, 126.
29. Freedman, H. H., Sultzer, B. M., and Kleinberg, W., unpublished data.
30. Reichard, S. M., Edelmann, A., and Gordon, A. S., Adrenal and hypophyseal influences upon the uptake of radioactive gold (Au^{198}) by the reticuloendothelial system, *Endocrinol.*, 1956, **59,** 55.
31. Jenkin, C. R., and Rowley, D., The role of opsonins in the clearance of living and inert particles by cells of the reticuloendothelial system, *J. Exp. Med.*, 1961, **114,** 363.

Downloaded from http://rupress.org/jem/article-pdf/116/6/929/1080888/929.pdf by guest on 09 August 2022