Toxic Lysolipid: Isolation from Pseudomonas pseudomallei

Abstract. *A lipoid of low molecular weight, isolated from the supernatant fluid of cultures of* Pseudomonas pseudomallei, *killed mice, but not rabbits, in doses comparable to those of the homologous lipopolysaccharide and also lysed sheep erythrocytes. In rabbits this lipoid failed to elicit primary dermal lesions, to prepare the skin for the local Schwartzman reaction, or to induce antibody formation.*

The precise relationships between the physicochemical constitution of bacterial endotoxins and their capacity to elicit characteristic biological reactions in mammals are the subject of controversy. It has been demonstrated, however, that removal of the protein moieties does not alter their biological properties (*1, 2*) and that appropriate acid hydrolysis yields phospholipids, which retain certain of the original endotoxic activities, and biologically inactive polysaccharides (*3, 4*). Whereas certain workers consider that the lipid moieties are responsible for many of the endotoxic reactions (*4, 5*), others give this view little support (*6*).

We have isolated a toxic and hemolytic lipoid from *Pseudomonas pseudomallei.* Although our starting material was not lipopolysaccharide itself, the presence of this lipoid in cultures of Gram-negative organisms may have bearing on the biologically active components of endotoxins.

Pseudomonas pseudomallei strain 118-6 was cultured for 3 days at 37°C in chemically defined media (*7*) and killed with 1 percent formalin at room temperature. Lipopolysaccharide was isolated from intact cells by initial phenol extraction (*1*), followed by fractionation with acetone and differential centrifugation; the lipoid, however, was obtained from the cell-free culture fluids. These supernatant fluids were stored at 4°C for about 9 months; a precipitate which formed was collected by centrifugation, dissolved in a mixture of acetone and distilled water (2:1 by volume), and filtered through a Whatman 41H paper. The preparation was dried under reduced pressure and dissolved in acetone. Insoluble material was removed by filtration through a Corning UF sintered glass filter. Distilled water (0.5 volume) was then added to the filtrate and the acetone removed by evaporation. The photograph on the cover shows the lipoid crystals that formed during the removal of the acetone. The crystals were dispersed at 55°C for 15 minutes and the preparation was dialyzed against distilled water for 3 days at 4°C.

The lipoid was also soluble in diethyl ether, methanol, ethanol, carbon disulfide, carbon tetrachloride, and chloroform. A 0.5-percent solution of the material in distilled water was highly opalescent and gave an acid reaction (*p*H 4.9). However, the solution cleared when the *p*H was adjusted to 7.2. In the ultracentrifuge, a solution of the lipoid in 0.03*M* phosphate buffer at *p*H 8 evinced a single symmetrical peak with a sedimentation coefficient of 1.85*S*. In contrast, the lipopolysaccharide at *p*H 8 produced an abnormally sharp boundary with a sedimentation coefficient of 43*S*. By electrophoresis at *p*H 8 it was demonstrated that both the lipoid and the lipopolysaccharide migrated as anions, with the lipoid having a greater mobility than the lipopolysaccharide (17.21 versus 6.77 $\times$ 10^{-5} cm^2 sec^{-1} $volt^{-1}$).

Toxicity was assayed in 3-, 4-, and 7-week-old mice of the Namru and Balb/C strains reared at 22°C. Graded doses of lipoid and lipopolysaccharide in 0.5 ml of distilled water, or of isotonic NaCl-tris buffer (*p*H 7.4), were inoculated intraperitoneally into groups of five mice each. The animals were observed for 5 days but more than 90 percent mortality occurred within 2 days of injection. As the toxicity of these materials was unaffected by their diluents, most titrations were done with the materials in distilled water because the lipopolysaccharide aggregated in the presence of electrolytes. Table 1 shows a representative titration in Balb/C mice. In both strains of mice, the toxicity of the lipoid approached that of the lipopolysaccharide. The pathognomonic picture of lipopolysaccharide toxicity—ocular discharge, muscular tremors, and myasthenia—was also that produced by the lipoid. However, lipoid toxicity was distinguishable by the absence of diarrhea and by the fact that lethal doses generally caused death within 3 hours as against 48 hours for the lipopolysaccharide.

In experiments with New Zealand rabbits, the lipoid, unlike the lipopolysaccharide, did not elicit primary dermal lesions or prepare the skin for the local Schwartzman reaction. This was so even when doses were 1000-fold higher than the minimum lipopolysaccharide dose effective for these reactions.

Intravenous injections of lipoid (1 mg/kg) into each of five rabbits, followed by 1-mg doses on days 3, 6, 15, and 22, were not lethal; these doses were 100 times the lethal dose of the lipopolysaccharide (*8*). The serums of the lipoid-treated rabbits did not react in agar diffusion tests with the lipoid or the lipopolysaccharide, nor did serums from rabbits, hyperimmunized with intact cells of the homologous organism, react with the lipoid. Negative reactions were also evinced in hemagglutination tests with serums from rabbits treated with lipoid and sheep erythrocytes sensitized with lipopolysaccharide.

During these studies we observed that the lipoid lysed sheep erythrocytes. Further experiments demonstrated that the lipoid acted specifically as a hemol-

Table 1. Assays in male Balb/C mice injected intraperitoneally with lipopolysaccharide and lipoid from *Pseudomonas pseudomallei* strain 118-6.

Age (wk)	Mice: Weight (g) Range	Mice: Weight (g) Mean	Total weight of materials injected (mg) 0.25	0.5	0.75	1.0	1.25	1.5	1.75	2.0
			Lipopolysaccharide							
4	12–16	14.01	0/5*	0/5	1/5	5/5	5/5			
4	12–16	14.62		1/5	2/5	1/5	5/5			
7	20–24	22.2	0/5	0/5		1/5	2/5	5/5	5/5	5/5
7	20–24	21.6		0/5	0/5	0/5	4/5	2/5	5/5	5/5
			Lipoid							
4	12–16	14.0			0/5	2/5	4/5	5/5		
4	12–16	14.6			0/5	0/5	0/5	5/5		
7	20–24	21.9		0/5		0/5		0/5	4/5	5/5
7	20–24	22.2		0/5		0/5		0/5	0/5	5/5

* Ratio of dead animals to total number injected.

ysin, since the respective lytic endpoints of 2.1 μg and 1.56 μg for 2-percent and 1-percent red blood cell suspensions were identical in both buffered and nonbuffered saline systems.

The results of these experiments demonstrated that the lipoid is sufficiently different from the lipopolysaccharide as not to be characterized, in the traditional sense, as endotoxin; and that its toxic effects cannot be ascribed to the presence of lipopolysaccharide. However, inasmuch as these two materials appear to be unrelated, the comparable lethal potencies in both Namru and Balb/C mice of the lipoid and lipopolysaccharide, together with certain similarities in their toxic action, are worthy of note. Although these data offer insufficient grounds for the conclusion that the lipid moiety of endotoxin is implicated in its lethal effect on mice, they do demonstrate that bacterial lipids can have significant toxic activity.

M. S. REDFEARN

University of California, Naval Biological Laboratory, Naval Supply Center, Oakland, California 94614

References and Notes

1. O. Westphal and O. Luderitz, *Angew. Chem.* **66**, 407 (1954).
2. M. E. Webster, J. G. Sagin, M. Landy, A. G. Johnson, *J. Immunol.* **74**, 455 (1955); M. Landy, A. G. Johnson, M. E. Webster, J. G. Sagin, *ibid.*, p. 466; E. Ribi, B. H. Hoyer, K. C. Milner, T. D. Perrine, C. L. Larson, G. Goode, *ibid.* **84**, 32 (1960).
3. A. Boivin, I. Mesrobeanu, L. Mesrobeanu, *Compt. Rend. Soc. Biol.* **114**, 307 (1933); W. R. J. Morgan and S. M. Partridge, *Biochem. J.* **34**, 169 (1940); C. G. Freeman, *ibid.* **37**, 601 (194→ W. F. Goebel, F. Binkley, E. Perlman, *J. Exptl. Med.* **81**, 315 (1945); A. Bendich and E. Chargaff, *J. Biol. Chem.* **166**, 283 (1946).
4. A. A. Miles and N. W. Pirie, *Brit. J. Exptl. Pathol.* **20**, 278 (1939).
5. E. Neter, H. Anzai, E. A. Gorzynski, A. Nowotny, O. Westphal, *Proc. Soc. Exptl. Biol. Med.* **103**, 73 (1960); E. Mihich, O. Westphal, O. Luderitz, E. Neter, *ibid.* **107**, 816 (1961); O. Westphal, *Ann. Inst. Pasteur* **98**, 789 (1960).
6. E. Ribi, W. T. Haskins, M. Landy, K. C. Milner. *J. Exptl. Med.* **144**, 647 (1961); E. Ribi, W. T. Haskins, M. Landy, K. C. Milner, *Bacteriol. Rev.* **25**, 427 (1961); W. T. Haskins, M. Landy, K. C. Milner, E. Ribi, *ibid.* **114**, 665 (1961); K. Fukushi, R. L. Anacker, W. T. Haskins, M. Landy, K. C. Milner, E. Ribi, *J. Bacteriol.* **87**, 391 (1964).
7. O. G. Lien, E. Isaeff, C. F. Tedford, *Univ. of California, Naval Biological Laboratory, Res. Stat. Rept. No. 18, App. B, Sect. VI* (1955).
8. G. J. Hildebrand, M. S. Redfearn, Y. Seys, *Proc. Soc. Exptl. Biol. Med.* **113**, 902 (1963).
9. I thank K. B. DeOme, Cancer and Genetics Laboratory, University of California, for the Balb/C mice, and also Mrs. Margaret Johnston for excellent technical assistance. This work was sponsored by the Office of Naval Research and the Bureau of Medicine and Surgery, United States Navy, under a contract between the Office of Naval Research and the Regents of the University of California.

30 April 1964

Proton Tunneling in Radiation-Induced Mutation

Abstract. *The equilibrium proton distribution and tunneling rate in the N–H . . . N hydrogen bond of the guanine-cytosine base pair have been calculated quantum mechanically for the ground state, a charge-transfer excited state, and positive and negative ionic states. These results are consistent with the idea that tautomeric rearrangement can be a cause of radiation induced mutation or carcinogenesis.*

It has long been recognized that the hydrogen bonds connecting the nucleotide bases of the two strands of the DNA molecule are crucially involved in the transfer and retention of genetic information. Under normal conditions, the bonds cause the bases of the two strands to be paired, guanine with cytosine (G–C) and adenine with thymine (A–T), so that the DNA replication process produces duplicates of the genetic coding of the replicating molecules. Improper pairing of the bases during replication would result in alteration of the genetic coding, thereby possibly producing mutations.

Watson and Crick (*1*) have suggested that the genetic coding may be perturbed by the presence of nucleotide bases appearing in the unusual, or "rare," tautomeric form. For example, the rare tautomer of guanine pairs with thymine instead of cytosine. Löwdin (*2*) pointed out that rare tautomers may be produced in pairs by proton rearrangements within the hydrogen bonds connecting a base pair, and that replications involving such rearranged base pairs would perpetuate genetic coding alterations.

We next consider the factors affecting proton rearrangements. The proton in a hydrogen bond is subject to a potential which in ordinary cases has two minima of unequal depth at the normal and tautomeric equilibrium positions, separated by a maximum which acts as a barrier to rearrangement. As indicated by Löwdin, the energy of the protonic motion is ordinarily less than the barrier height, and the proton may get from one minimum to the other by quantum mechanical tunneling. The tunneling process is very sensitive to the shape of the barrier, and particularly to its height and breadth. This suggests that mutagens, and possible carcinogens, may act by affecting the protonic potential, and particularly the barrier.

One way in which the protonic potential can be affected is by forming electronically excited, or ionized, states. Several calculations have been made of the π-electronic structure of the ground and excited states of nucleotide base pairs (*3*, *4*). Rein and Ladik (*4*) found, from an approximate self-consistent field calculation, that a charge-transfer excited state of the G–C base pair seemed relatively favorable for a proton rearrangement. The ground state, and all states considered for the A–T pair, appeared less favorable. Thus, radiation capable of producing this excited state would be expected to have a significant influence on the production of tautomeric base pairs and, thence, mutations. In the absence of radiation, rearrangements would have to take place in the ground electronic state. Löwdin has remarked that in that case the energetically most favorable process may be the simultaneous tunneling of two hydrogen bonds in the G–C base pair.

We have extended the study of the proton rearrangements by making calculations of the protonic potential function by methods which consider both the π-electrons of the bases and the σ-electrons directly involved in the hydrogen bonding. We have obtained approximate self-consistent field solutions for the potential of the N–H . . . N bond of the G–C base pair in its ground state, and several excited states and ionic forms. The excited states are subject to rather severe approximations, but we believe they are nevertheless qualitatively useful. Full descriptions of the quantum mechanical methods and calculations will appear elsewhere (*5*). By certain methods, conveniently summarized by Löwdin (*6*), we have derived from the protonic potential for each electronic state the equilibrium proton distribution among the tautomeric

Table 1. Tunneling time and tautomeric equilibrium constant of the ground, excited, and ionic states of the guanine-cytosine base pair; K is equal to the ratio of C_{rare} to C_{normal} where C is the population number.

State	τ (sec)	K
Ground	2.7×10^{-10}	2.4×10^{-23}
Excited	6.2×10^{-6}	2.3×10^{-5}
Positive ion	3.3×10^{-4}	3.1×10^{-2}
Negative ion	1.2×10^{-6}	8.0×10^{4}