# Studies on a Lipopolysaccharide from *Escherichia coli*. Heterogeneity and Mechanism of Reversible Inactivation by Sodium Deoxycholate*

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ABSTRACT: A lipopolysaccharide from *Escherichia coli* K-235 contains free amino groups which can be converted into the 2,4-dinitrophenylamino groups without loss of pyrogenicity. This derivative permitted study of its reversible disaggregation and inactivation by sodium deoxycholate at low concentrations of both solutes; 99% inactivation of pyrogenicity required at least 1 mg of sodium deoxycholate/ml and was accompanied by 100% disaggregation of 2,4-dinitrophenylaminolipopolysaccharide to a subunit of 118,000 daltons.

The same degree of disaggregation to the same size of subunit was also obtained without any decrease in pyrogenicity in solutions of 0.02 and 0.03 mg of sodium deoxycholate per ml. Regardless of the sodium deoxycholate concentration 100% disaggregation required a sodium deoxycholate:2,4-dinitrophenylaminolipopolysaccharide ratio of only 4:6, but 0.03 mg of sodium deoxycholate/ml failed to depress pyrogenicity even though the sodium deoxycholate:2,4-dinitrophenylaminolipopolysaccharide ratio was 3000. Inactivation of 2,4-dinitrophenylaminolipopolysaccharide therefore probably depends upon an interaction with sodium deoxycholate beyond that which is required for disaggregation. Gel permeation chromatography in sodium deoxycholate solution indicated that lipopolysaccharide is a heterogeneous population of closely related molecules which are all highly pyrogenic.

Sodium deoxycholate interacts reversibly with lipopolysaccharides from gram-negative bacteria (LPS,[1] endotoxins) to greatly decrease several of their biological properties, such as pyrogenicity, immunogenicity, and the immunological adjuvant effect. This has been demonstrated adequately (Ribi *et al.*, 1966; Rudbach *et al.*, 1967; Tarmina *et al.*, 1968a,b; Jackson, 1969) and it has been proposed that sodium deoxycholate causes LPS aggregates to dissociate into biologically inactive subunits, with a molecular weight around 20,000. Working with LPS from a different bacterium, we have confirmed the 100-fold decrease in pyrogenicity when LPS is dissolved in sodium deoxycholate solution, and in an earlier paper (McIntire *et al.*, 1967) we took the position that disaggregation to inactive subunits may not be the mechanism of this effect.

The centrifugation data (Ribi *et al.*, 1966) which were the basis for the "inactive subunit" concept were obtained at concentrations of sodium deoxycholate where we have encountered extensive formation of micelles with $s_{20,w}$ values around 1.0 S. Because of concern over the feasibility of measuring the hydrodynamic properties of LPS in the presence of sodium deoxycholate micelles, we devised a way of studying the disaggregation without this complication. We have labeled LPS with 2,4-dinitrophenyl groups, and, by using this derivative in the analytical ultracentrifuge equipped with split-beam photoelectric scanning optics, we have been able to study its disaggregation in concentrations of sodium deoxycholate below the micelle-forming range. Also we have attempted to use gel permeation chromatography as an indication of the molecular size of LPS in sodium deoxycholate solution.

## Materials and Methods

The preparation of LPS from *Escherichia coli* K235 and most of our analytical methods were described previously (McIntire *et al.*, 1967).

*Dinitrophenylaminolipopolysaccharide (DNP-LPS).* LPS (1 g) was suspended in 250 ml of half-saturated sodium acetate in an ice bath and the pH was adjusted to 9 with sodium hydroxide; 200 μl of bromoacetyl bromide dissolved in 10 ml of dry reagent grade dioxane was added dropwise, and simultaneously 1 N NaOH was added at a rate sufficient to maintain the pH between 8.5 and 9.5. After addition of all the bromoacetyl bromide, the pH was adjusted to approximately 4.5 with 6–12 N HCl. The preparation was dialyzed for 5–7 days at 4° in a cloth-jacketed Visking tube. The bromoacetylated product (0.2 g) was allowed to react with an equal weight of *N*-(2,4-dinitrophenyl)ethylenediamine at pH 9 for 12–20 hr at room temperature, with constant stirring. The solution was adjusted to pH 4 and dialyzed exhaustively against $10^{-4}$ N HCl; the amount of *N*-(2,4-dinitrophenyl)ethylenediamine bound was determined by the absorbance at 340 mμ.

*Chromatography.* LPS and DNP-LPS were chromatographed on Sephadex G-100 which was equilibrated and eluted with 1% sodium deoxycholate solution at pH 8.1. The column effluent was examined for 220-mμ absorbancy and reaction with anthrone reagent.

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[1] Abbreviations used are: LPS, lipopolysaccharide; DNP-LPS, 2,4-dinitrophenylaminolipopolysaccharide.

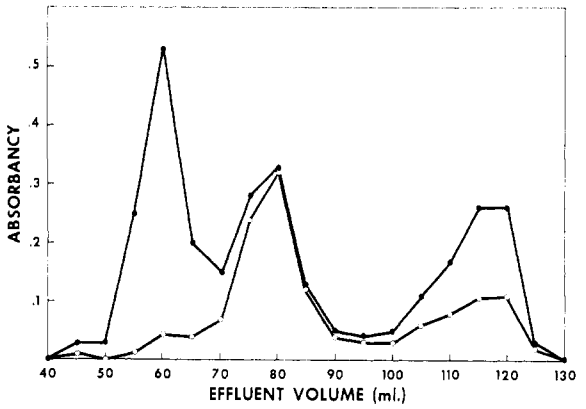


FIGURE 1: Chromatographic pattern of LPS on Sephadex G-100 with 1% sodium deoxycholate eluent. Absorbancy was measured at 220 mμ for upper curve and 625 mμ for anthrone reaction product aliquot in lower curve.

*Amino Nitrogen.* The ninhydrin method of Rosen (1957) was used.

*Sugars, Anthrone.* Sucrose was used as a reference carbohydrate in the anthrone reaction (Kabat and Mayer, 1961).

*Sedimentation Equilibrium.* All sedimentation equilibrium runs were made on a Spinco Model E ultracentrifuge equipped with split-beam photoelectric scanning optics (Schachman *et al.*, 1962; Lamers *et al.*, 1963). Scans were made on the DNP-LPS preparations at 350 mμ. Runs were made at 9000 rpm using both 12- and 30-mm double-sector cells. All runs were at or near 15° using the techniques to prevent convection described by Schachman and Edelstein (1966), and were n 0.2 M Tris-Cl buffer (pH 8.0).

The per cent subunit was determined by integrating the area under the $C_0$ scan as the run reached speed and then intergrating the area under the equilibrium scan. It was assumed that any difference between these areas was a result of spinning large aggregates out of solution.

The molecular weights ($M_w$, weight average) were calculated from the equilibrium data by the equation, $M_w = (2RT/(1 - \bar{v}\rho)w^2)(\mathrm{d} \ln c/\mathrm{d}r^2)$, where $c$ is directly proportional to the recorder deflection of the scanner. The $M_z$ values were calculated from the scanner plot

$$M_z = \frac{RT}{(1 - \bar{v}\rho)w^2} \frac{\frac{1}{b}\left(\frac{\mathrm{d}c}{\mathrm{d}r}\right)_b - \frac{1}{a}\left(\frac{\mathrm{d}c}{\mathrm{d}r}\right)_a}{c_b - c_a}$$

where $(\mathrm{d}c/\mathrm{d}r)_b$ and $(\mathrm{d}c/\mathrm{d}r)_a$ are computer-derived concentration gradients at the solution bottom and meniscus, respectively.

In all runs, after equilibrium was reached and final scans were made, the centrifuged was accelerated to maximum speed to determine a true base line and to correct for small contaminants in the solution (Barlow *et al.*, 1969). This correction was relatively small.

The partial specific volume, $\bar{v}$, determined pycnometrically in 0.15% sodium deoxycholate, was 0.66 ± 0.01; 0.66 was used in all calculations.

Multiple determinations of molecular weight with 53 μg of DNP-LPS/ml in 0.15% sodium deoxycholate showed reproducibility to about 118,000 ± 5,000.

TABLE I: Comparison of LPS Fractions.

| | Molecular Weight | | |
|---|---|---|---|
| | 118,000 I, % | 113,000 II, % | 118,000 III, % |
| Sugars, anthrone | 22 | 32 | 15 |
| Phosphorus | 1.85 | 1.25 | 3.0 |
| Nitrogen | 1.85 | 2.05 | 1.39 |
| Amino nitrogen | 0.073 | 0.084 | 0.16 |
| Lauric acid | 3.5 | 1.1 | 5.3 |
| Myristic | 2.5 | 1.1 | 3.2 |
| β-Hydroxymyristic acid | 8.4 | 4.2 | 12.7 |

## Results

*Heterogeneity of LPS.* Chromatography on Sephadex G-100 of LPS dissolved in 1% sodium deoxycholate was carried out in the hope of obtaining a suggestion as to the molecular size of LPS in a concentration of sodium deoxycholate sufficient to block 99% of the pyrogenicity. Although the LPS preparation used had shown only a single component with no indication of heterogeneity when chromatographed in 2.5% sodium dodecyl sulfate solution on Sephadex G-100 (McIntire *et al.*, 1967), three major components became evident when the chromatography was performed in the presence of 1% sodium deoxycholate, as shown in Figure 1. When the fractions under the main portion of each peak were pooled and rechromatographed, each pool appeared as a single component in the same position as before. For Figure 1, 30 mg of LPS was chromatographed on a 2.5 × 36 cm column; similar results were obtained with 500 mg on a 1.9 × 145 cm column; LPS and DNP-LPS behaved identically.

While the chromatography indicated that the three components differed in molecular weight, and calibration of the column with molecules of known size in the presence of 1% sodium deoxycholate indicated that peak III was in the range of 10,000–20,000, equilibrium sedimentation showed that all three components were essentially the same size, 113,000–118,000 (Table I). That peak III in 1% sodium deoxycholate solution had a molecular weight greater than 50,000 was indicated also by its failure to pass through an XM-50 Diaflow membrane (Amicon Corp., Cambridge, Mass.). This great discrepancy between the column and the sedimentation data on the molecular weight of peak III demonstrate that gel permeation chromatography can be very misleading.

That the three major fractions of LPS are indeed qualitatively different is shown by the analytical data of Table I and by the electrophoretic mobility (Figure 2). As regards antigenic specificity, it was possible to neutralize anti-LPS serum completely with each component so that the neutralized antiserum would not show a reaction with either of the other components in immunodiffusion plates. However, the three components differed greatly in the quantity required to neutralize a given amount of antiserum. The quantities of I, II, and III required were in a ratio of 8:1:32. Differences among the three components were apparent in quantitative precipitin curves (Figure 3). All three components of LPS were highly pyrogenic.

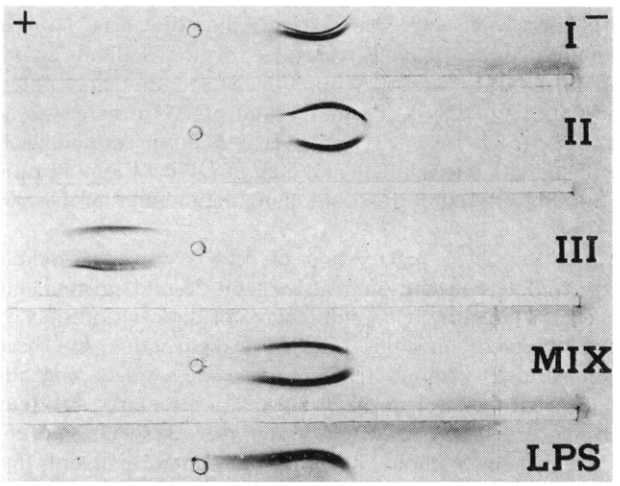


FIGURE 2: Agar immunoelectrophoresis pattern of LPS and fractions I, II, and III. Samples were in 0.15% sodium dexoycholate.

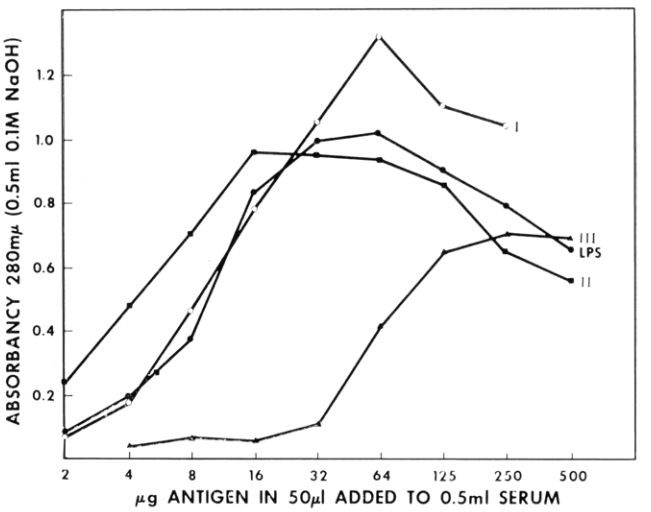


FIGURE 3: Quantitative precipitin analysis of LPS and fractions I, II, and III. LPS and fraction I were solubilized with an equal weight sodium dodecyl sulfate.

In the sedimentation studies components II and III showed no obvious heterogeneity. While component I had a weight average of 113,000, in agreement with II and III, the $M_z$ of this component was 166,000; this indicates a considerable degree of heterogeneity.

Thus far we have examined only two other lipopolysaccharides by gel permeation chromatography in the presence of 1% sodium deoxycholate. A preparation from *Salmonella enteritidis*, purchased from Difco and purified in our laboratory, dissociated into three components with a pattern similar to Figure 1. A preparation from *Aerobacter aerogenes*, kindly supplied by Professor J. F. Snell of Ohio State University, gave a small component analogous to I, but nearly all of the preparation moved similarly to component II of Figure 1.

*Concentration of Sodium Deoxycholate Required for Reversible Inactivation.* For studying the concentration of sodium deoxycholate required for inactivation of LPS, and the apparent molecular size in relation to sodium deoxycholate concentration, we chose component III from the chromatography of DNP-LPS in 1% sodium deoxycholate solution on Sephadex G-100. The pyrogenicity was totally restored upon removal of sodium deoxycholate by dialysis, and was not affected by the few DNP groups attached to LPS. We wished to determine the relationship between the concentration of sodium deoxycholate in the solution injected into the rabbit, and the degree of inhibition of the pyrogen reaction. The data of Table II indicate that 1.5 mg of sodium deoxycholate/ml gives approximately 99% inhibition; the lowest concentration of sodium deoxycholate at which we have observed this degree of inhibition is 1 mg/ml, and at 0.03 mg of sodium deoxycholate/ml there is no inhibition. These observations are essentially in agreement with those of Ribi *et al.* (1966).

*The Effect of Sodium Deoxycholate Concentration on Disaggregation of DNP-LPS.* To eliminate any effects of sodium deoxycholate micelles in the sedimentation experiments we chose to work only at sodium deoxycholate concentrations below the critical level for micelle formation in 0.2 M Tris-Cl buffer (pH 8.0). By sedimentation and light-scattering criteria 1.5 mg of sodium deoxycholate/ml proved to be the maximum concentration suitable for our purpose. This level of sodium

TABLE II: The Effect of Sodium Deoxycholate Concentration on Pyrogenicity of DNP-LPS.

| DNP-LPS (μg/ml) | Sodium Deoxycholate (mg/ml) | Sodium Deoxycholate: DNP:LPS | No. of Rabbits | Av $\Delta T^a$ |
|---|---|---|---|---|
| 0.01 | | | 18 | 1.7 |
| 0.001 | | | 15 | 0.4 |
| 1.0 | 1.5 | 1,500 | 9 | 1.1 |
| 0.1 | 1.5 | 15,000 | 9 | 0.6 |
| 0.01 | 0.1 | 10,000 | 9 | 1.2 |
| 0.01 | 0.03 | 3,000 | 3 | 1.5 |
| 0.01 | 0.02 | 2,000 | 3 | 2.3 |

[a] Temperature increase, 3 hr after injection.

TABLE III: The Effect of Sodium Deoxycholate Concentration and Sodium Deoxycholate:DNP-LPS Ratio on the Disaggregation of DNP-LPS.

| DNP-LPS (μg/ml) | Sodium Deoxycholate (mg/ml) | Sodium Deoxycholate: DNP-LPS | % Monomer | Monomer Mol Wt |
|---|---|---|---|---|
| 53.0 | 1.5 | 28 | 100 | 118,000 |
| 53.0 | 0.1 | 1.9 | 70 | 115,000 |
| 53.0 | 0.02 | 0.4 | 5 | |
| 10.0 | 0.1 | 10 | 100 | 108,000 |
| 10.0 | 0.02 | 2 | 65 | 102,000 |
| 5.0 | 0.03 | 6 | 100 | 118,000 |
| 5.0 | 0.02 | 4 | 94 | 118,000 |

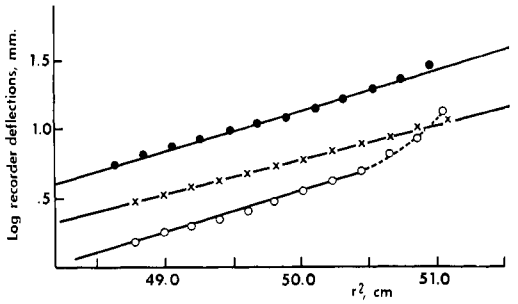


FIGURE 4: Sedimentation equilibrium data on DNP-LPS under various conditions. (●) 53 μg of DNP-LPS and 1.5 mg of sodium deoxycholate/ml in 12-mm cell. (×) 10 μg of DNP-LPS and 0.1 mg of sodium deoxycholate/ml in 30-mm cell. (○) 10 μg of DNP-LPS and 0.02 mg of sodium deoxycholate /ml in 30-mm cell.

deoxycholate nullifies the pyrogenicity of either LPS or DNP-LPS by approximately 99% and causes a total dissociation of DNP-LPS to subunits with a molecular weight of 118,000. Table III shows the molecular weight and the per cent of subunit in different concentrations of sodium deoxycholate and at different ratios of sodium deoxycholate:DNP-LPS. Figure 4 shows typical plots of the equilibrium sedimentation data from which the molecular weights and percentages of subunit were calculated. The degree of curvature toward the bottom of the cell (right side of the figure) is proportional to the amount of material larger than the subunit. These data indicate that the degree of dissociation is a function of the ratio of sodium deoxycholate:DNP-LPS, and that a ratio of 4:6 is sufficient for 100% dissociation to a subunit of 118,000 daltons. The importance of this ratio is valid, and the size of subunit is constant over a wide range of concentrations of both sodium deoxycholate and DNP-LPS.

## Discussion

In view of the simple and unsophisticated procedure for the isolation of LPS, the heterogeneity shown by chromatography in sodium deoxycholate solution is not at all surprising, even though earlier attempts to demonstrate heterogeneity had failed (McIntire *et al.*, 1967). Indeed we are prepared to learn that components I, II, and III are really not homogeneous but are only populations of closely related molecules. The known differences among I, II, and III do not offer any new suggestion as to a relationship between molecular composition and pyrogenicity, because all three fractions were highly pyrogenic, with no striking differences in potency.

The main point of interest in this paper is the mechanism by which sodium deoxycholate inactivates DNP-LPS reversibly. Does sodium deoxycholate cause the dissociation of DNP-LPS to subunits which are intrinsically inactive, or is an additional interaction between the two molecules required? The data presented in Tables II and III indicate that the same degree of dissociation and the same size of subunit were obtained, both in low concentrations of sodium deoxycholate which showed no inactivation and in higher concentrations of sodium deoxycholate which gave approximately 99% inactivation of DNP-LPS. Moreover, the degree of dissociation was independent of sodium deoxycholate concentration as long as the sodium deoxycholate:DNP-LPS ratio was 4:6 or greater, while 99% inactivation required at least 1 mg of sodium deoxycholate/ml regardless of the sodium deoxycholate:DNP-LPS ratio. With 30 μg of sodium deoxycholate and 0.01 μg of DNP-LPS/ml (a ratio of 3000) there was no inhibition of pyrogenicity, and yet a solution containing 30 μg of sodium deoxycholate and 5 μg of DNP-LPS/ml (a ratio of 6) showed 100% dissociation to the subunit of 118,000 daltons.

Thus the 99% inactivation of LPS requires a sodium deoxycholate concentration at least 30–50-fold greater than is necessary for the maximum degree of dissociation that we have observed, and this degree of dissociation has been demonstrated without inactivation. These facts cogently suggest that the mechanism of inactivation requires a sodium deoxycholate–LPS interaction beyond mere dissociation of the LPS aggregate. Without more information we can only hypothesize the formation of an inactive, reversible sodium deoxycholate–LPS complex which requires a certain level of sodium deoxycholate for stability, and in which the loss of biological activity may be the result of a change in the conformation of LPS.

These conclusions are based on experiments with a lipopolysaccharide from only one species of gram-negative bacteria and may, therefore, have obvious limitations. Our studies were made possible by the sensitivity of the photoelectric scanner optics for the ultracentrifuge, and by our ability to attach the 2,4-dinitrophenylamino group to the LPS molecule without changing the pyrogenicity. When more sensitive tools are available it may be possible to study the dissociation of LPS at even lower concentrations than we were able to use.

This paper is not concerned with the question of *ultimate* subunits of LPS. A subunit as small as 65,000 daltons has been observed as a result of treatment of an LPS from *Aerobacter vinelandii* with a combination of sodium dodecyl sulfate and EDTA (Olins and Warner, 1967). Possibly such a treatment of our LPS would yield a subunit of similar size.

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## References

Barlow, G. H., Summaria, L., Robbins, K. C. (1969), *J. Biol. Chem. 244*, 1138.

Jackson, A. L. (1969), *J. Bacteriol. 97*, 13.

Kabat, E. A., and Mayer, M. M. (1961), Experimental Immunochemistry, Springfield, Ill., C. C Thomas, p 528.

Lamers, K., Putney, F., Steinberg, I. Z., and Schachman, H. K. (1963), *Arch. Biochem. Biophys. 103*, 379.

McIntire, F. C., Sievert, H. W., Barlow, G. H., Finley, R. A., and Lee, A. Y. (1967), *Biochemistry 6*, 2363.

Olins, A. L., and Warner, R. C. (1967), *J. Biol. Chem. 242*, 4994.

Ribi, E., Anacker, R. L., Brown, R., Haskins, W. T., Malmgren, B., Milner, K. C., and Rudbach, J. A. (1966), *J. Bacteriol. 92*, 1493.

Rosen, H. (1957), *Arch. Biochem. Biophys. 67*, 10.

Rudbach, J. A., Milner, K. C., and Ribi, E. (1967), *J. Exptl. Med. 126*, 63.
Schachman, H. K., and Edelstein, S. J. (1966), *Biochemistry 5*, 2681.
Schachman, H. K., Gropper, L., Hanlon, S., and Putney, F. (1962), *Arch. Biochem. Biophys. 99*, 175.
Tarmina, D. F., Milner, K. C., Ribi, E., and Rudbach, J. A. (1968a), *J. Immunol. 100*, 444.
Tarmina, D. F., Milner, K. C., Ribi, E., and Rudbach, J. A. (1968b), *J. Bacteriol. 96*, 1611.

# Phytol-Derived $C_{19}$ Di- and Triolefinic Hydrocarbons in Marine Zooplankton and Fishes*

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ABSTRACT: Three phytol-derived olefinic hydrocarbons have been isolated from marine zooplankton and fishes. Their structures have been determined by ultraviolet, infrared, nuclear magnetic resonance, and mass spectrometry and by combined gas chromatography and mass spectrometry of their ozonolysis products. They are the 2,10- and 5,10-diene and the 2,6,10-triene analogs of pristane (2,6,10,14-tetramethylpentadecane). The presumed mode of formation of these and related olefins and their fate in the marine food chain and in marine sediments is discussed. Because of their relative stability, these and related hydrocarbons provide tracers for the study of dynamic processes in the marine food chain. These olefins are not present in ancient sediments and in petroleum; therefore, they are valuable markers for the distinction between marine hydrocarbons derived from organisms and from oil pollution.

Chromatographic analysis of the lipids from marine zooplankton and from fish livers has yielded a saturated hydrocarbon fraction, consisting of pristane (2,6,10,14-tetramethylpentadecane, I) (Blumer *et al.*, 1963, 1964) with traces of normal alkanes and an unsaturated fraction containing squalene (Blumer, 1967) and a complex mixture of $C_{19}$ and $C_{20}$ olefins. We have previously reported the structures of three $C_{19}$ monoolefins (II–IV) (Blumer and Thomas, 1965b) and four $C_{20}$ diolefins (V–VIII) (Blumer and Thomas, 1965a). We wish to report the structures of three closely related $C_{19}$ di- and triolefins (IX–XI). The gas chromatographic retention indices of I–XI are listed in Table I to facilitate their determination in other natural products.

The three unknown olefins have been identified in mixed zooplankton from the Gulf of Maine. They occur in *Calanus finmarchicus*, *Calanus hyperboreus*, and *Calanus glacialis;* the same compounds have been found in the liver of the herring (*Clupea harengus L.*), of the basking shark (*Cetorhinus maximus Gunnerus*) and, commonly, in other members of the marine food chain. Because of their low concentration in marine lipids, between 1 and 50 ppm, isolation in sufficient quantities for structural elucidation is difficult. A better source is crude pristane this is obtained commercially by molecular distillation of basking shark lipids. From that source we have isolated the three corresponding olefins. Their gas chromatographic retention indices agree with those of the olefins in native basking shark liver oil and in zooplankton to better than $\pm 2$ units (see Table I). If isomerization of the olefins had occurred during preparation of the commercial pristane, a large shift in retention indices, especially on the more polar columns would be evident. The identity of the olefins from the three sources is conclusively proven by their identical retention parameters in liquid and gas chromatography.

The olefins are concentrated by frontal analysis of the crude pristane over deactivated silica gel; the concentrates are further separated by repeated adsorption chromatography on the same adsorbent. Again, gas chromatography on several substrates was used to ensure the identity of the products with the starting material; this insures against the possibility that the olefins might be artifacts produced on the adsorbent, *e.g.*, by isomerization. Complete chromatographic resolution, especially of the two isomeric dienes, is difficult and is best achieved by trapping of effluent samples from an analytical gas chromatography column. A strongly polar column (*e.g.*, FFAP) of at least 3000 theoretical plates is necessary for the resolution.

Molecular weights of the three unknowns were obtained by mass spectrometry; IX and X are isomeric $C_{19}$ diolefins (mol wt 264), XI is a $C_{19}$ triolefin (mol wt 262). This agrees with their gas chromatographic behavior and the greater $\Delta I$ for XI on the most polar columns (Kovats, 1958; Wehrli and Kovats, 1959). Because of hydrogen rearrangement during excitation, the mass spectra are inadequate to locate the position of the double bonds. This situation is commonly encountered for alkenes not containing tetrasubstituted double bonds.

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