Phospholipids of *Haemophilus influenzae* Rd during Exponential Growth and following the Development of Competence for Genetic Transformation

By SARAH L. SUTRINA AND J. J. SCOCCA

*Department of Biochemical and Biophysical Sciences,
The Johns Hopkins University School of Hygiene and Public Health,
Baltimore, Maryland 21205, U.S.A.*

(Received 14 August 1975; revised 6 October 1975)

INTRODUCTION

In studies on the membrane constituents of *Haemophilus influenzae* Rd, we determined the phospholipid content of the organism and identified the phospholipids of *H. influenzae* as phosphatidyl ethanolamine (85 %) and phosphatidyl glycerol (15 %). Development of the ability to take up external transforming DNA (competence) did not affect this proportion.

METHODS

Cells were grown and made competent by the two-stage procedure of Herriott, Meyer & Vogt (1970); the P_i content of the stage I medium (heart infusion) was decreased to 0.4 μ mol/ml as described by Scocca, Poland & Zoon (1974), and the P_i concentration of the stage II medium (M IV) was adjusted to this value. Sufficient $^{32}P_i$ was added to give a specific activity of 5 Ci/mol. Organisms grown and made competent in these media yielded 1 % transformants to streptomycin resistance, indicating that nearly all the bacteria were capable of taking up DNA. Labelled cells were harvested by centrifuging and washed with 0.15 M-NaCl; the phospholipids were extracted by a modification (Bertsch, Bonsen & Kornberg, 1969) of the Bligh & Dyer (1959) procedure, and concentrated by evaporation under N_2 .

Resolution of individual phospholipid classes was accomplished by thin-layer chromatography on silica gel plates (Chromagram polyvinyl alcohol binder, Eastman). Solvent A was chloroform-methanol-acetic acid (65:25:8, by vol.) and solvent B was chloroform-methanol-water (65:25:4, by vol.). Cardiolipin, phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine were purchased from Calbiochem and phosphatidyl glycerol and [^{32}P]cardiolipin were prepared from *Escherichia coli* B by preparative thin-layer chromatography. Reagents were made to show up either by charring with 30 % (v/v) H_2SO_4 , by exposure to I_2 vapour, by Rhodamine 6G fluorescence, or with ninhydrin (for free amino groups). Labelled phospholipids were located by autoradiography, and quantified by counting the appropriate excised regions of the chromatograms. Mobilities on thin-layer plates were expressed relative to the mobility of cardiolipin, and designated R_{Ci} . The relative mobilities of phosphatidyl ethanolamine and of cardiolipin under our conditions differed substantially from those reported elsewhere (e.g. Ames, 1968), possibly because we used different thin-layer plates. All procedures used gave consistent results both with standard reagents and with phospholipid extracts from *E. coli*.

Table 1. Identification of phospholipids from exponential-phase *H. influenzae* by t.l.c.

Phospholipid	R_{Cl} *		Ninhydrin reaction	Rhodamine 6G fluorescence
	Solvent A	Solvent B		
Cardiolipin	1.00	1.00	—	Red
Phosphatidyl ethanolamine	0.73	1.10	+	Yellow
Phosphatidyl glycerol	0.88	0.87	—	Blue
Phosphatidyl choline	0.28	0.65	—	Yellow
Phosphatidyl serine	0.45	0.65	+	ND
<i>H. influenzae</i> phospholipids				
Major component	0.73	1.10	+	Yellow
Minor component	0.89	0.88	—	Blue

* Mobility relative to cardiolipin. ND, not determined.

Table 2. Identification of the alkaline deacylation products from *H. influenzae* phospholipids

Mobilities were determined using [the solvent systems of Dawson (1960). Solvent 1 is phenol saturated with H₂O-acetic acid-ethanol (100:10:12, by vol.) Solvent 2 is methanol-formic acid (88 %, v/v)-H₂O (160:29:11). Standards were located by spraying with acid-molybdate; radioactive *H. influenzae* products were located by using a recording strip counter.

Compound	R_F	
	Solvent 1	Solvent 2
Glycerophosphorylglycerol	0.48	0.51
Glycerophosphorylethanolamine	0.62	0.38
Glycerophosphorylserine	0.38	—
Glycerophosphorylcholine	0.92	0.60
Major <i>H. influenzae</i> product	0.60	0.37
Minor <i>H. influenzae</i> product	0.46	0.51

The identities of phospholipids were also established by comparison of deacylation products prepared by mild alkaline hydrolysis (Wells & Dittmer, 1966). The water-soluble products were identified by ascending chromatography on Whatman No. 1 paper (washed with H₂O), using two solvent systems (Dawson, 1960).

RESULTS AND DISCUSSION

The phospholipid fraction from both exponential-phase (density 10⁹ bacteria/ml) and competent *H. influenzae* accounted for 10 to 11 % of the total dry weight of the cells, and was 0.16 mg/mg protein. The phospholipid content of cultures after development of competence increased 1.5-fold, thus paralleling the increase in protein in these cultures (Miller, 1970).

Chromatographic analyses of the phospholipids of exponential-phase *H. influenzae* revealed the presence of two components (Table 1): the major phospholipid was phosphatidyl ethanolamine, 85 % of the total, and the minor component was phosphatidyl glycerol, 15 %. Substantial over-exposure of autoradiograms did not reveal additional components, indicating that no other phospholipid accounted for more than 0.1 % of the total fraction.

The identification of the phospholipids was confirmed by comparison of the deacylated derivatives of the *H. influenzae* phospholipids with authentic standards. The results (Table 2), confirm the identification of the major component as phosphatidyl ethanolamine, and the lesser one as phosphatidyl glycerol.

In view of our failure to observe cardiolipin in these extracts, and because of a report that *H. influenzae* possesses a powerful phospholipase D specific for cardiolipin (Ono & White, 1970), we mixed [^{32}P]cardiolipin with a non-radioactive pellet of *H. influenzae* and extracted the mixture. The added radioactivity was quantitatively recovered in the lipid fraction, and migrated as cardiolipin on t.l.c. The presence of cardiolipin in extracts of *H. parainfluenzae* was readily demonstrated. These findings, together with the absence of phosphatidic acid in extracts of *H. influenzae*, indicate that cardiolipin was stable in our extraction procedures and that it was absent from the membranes of *H. influenzae*.

The phospholipids synthesized during development of competence were identical to those made during exponential growth. Phosphatidyl ethanolamine accounted for $85.4 \pm 1.5\%$ (mean $\pm$ standard deviation for five determinations) of the total phospholipid labelled during growth, and $85.0 \pm 1.5\%$ of the phospholipid labelled during development of competence in radioactive medium; the remaining fraction in both cases was phosphatidyl glycerol. Identical results were obtained when the organisms were labelled during growth and allowed to develop competence in non-radioactive medium.

Thanks are due to S. S. Krag and K. C. Zoon for their advice and to K. Moore for assistance in preparing the manuscript. This work was supported by the American Cancer Society (grant NP 121). S. L. S. is a Worthington-Kunitz Fellow; J. J. S. has a Public Health Service Career Development Award from the National Institute of General Medical Sciences (K4-GM 70663).

REFERENCES

- AMES, G. F. (1968). Lipids of *Salmonella typhimurium* and *Escherichia coli*: structure and metabolism. *Journal of Bacteriology* **95**, 833-843.
- BERTSCH, L. L., BONSEN, P. P. M. & KORNBERG, A. (1969). Biochemical studies of bacterial sporulation and germination. XIV. Phospholipids of *Bacillus megaterium*. *Journal of Bacteriology* **98**, 75-81.
- BLIGH, E. G. & DYER, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911-917.
- DAWSON, R. M. C. (1960). A hydrolytic procedure for the identification and estimation of individual phospholipids in biological samples. *Biochemical Journal* **74**, 45-53.
- HERRIOTT, R. M., MEYER, E. M. & VOGT, M. (1970). Defined non-growth media for stage II development of competence in *Haemophilus influenzae*. *Journal of Bacteriology* **101**, 517-524.
- MILLER, D. H. (1970). *The involvement of inosine in the regulation of competence development in Haemophilus influenzae*. Dissertation, The Johns Hopkins University, Baltimore.
- ONO, Y., & WHITE D. C. (1970). Cardiolipin-specific phospholipase D activity in *Haemophilus parainfluenzae*. *Journal of Bacteriology* **103**, 111-115.
- SCOCCA, J. J., POLAND, R. L. & ZOON, K. C. (1974). Specificity in deoxyribonucleic acid uptake by transformable *Haemophilus influenzae*. *Journal of Bacteriology* **118**, 369-373.
- WELLS, M. A. & DITTMER, J. C. (1966). A microanalytical technique for the quantitative determination of twenty-four classes of brain lipids. *Biochemistry* **5**, 3405-3418.