Brit. J. vener. Dis. (1966), **42,** 125.

CHEMICAL STRUCTURE AND SEROLOGICAL ACTIVITY OF NATURAL AND SYNTHETIC CARDIOLIPIN AND RELATED COMPOUNDS*

BY

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Since its first isolation from ox heart by Pangborn (1941), cardiolipin has found general application in the sero-diagnosis of syphilis. Mixtures of cardiolipin, lecithin, and cholesterol have replaced the former tissue extracts as antigens in flocculation and complement-fixation tests. International reference preparations of cardiolipin and of lecithin are being distributed under the supervision of WHO and analytical requirements for these substances have been published in the International Pharmacopoeia (1955). Occasionally, however, analytical data of serologically satisfactory batches are not within the required range†. Inasmuch as natural compounds generally have a certain variation in composition, the use of synthetic products would be advantageous from the point of view of standardization.

In the first part of this paper, literature regarding the chemical structure of cardiolipin and the serological activity of similar synthetic compounds is reviewed. It will be seen that investigations did not result in the preparation of an entirely satisfactory substitute for cardiolipin.

Recently, de Haas and van Deenen (1965a) succeeded in synthetizing a diphosphatidylglycerol. The serological activity of their product has been studied in our Institute and the results are reported in the second part of this paper.

Review of Literature

Chemical Structure of Natural Cardiolipin

Soon after its first isolation, cardiolipin was identified as a complex phosphatidic acid sodium salt with a sodium:phosphorus ratio of 1:1 (Pangborn, 1942, 1944). Comparison between analytical data of products obtained by alkaline hydrolysis and those calculated for tentative formulae, led Pangborn (1947) to the structure given in Fig. 1. Assuming the six available hydroxyl groups to be esterified with linoleic and oleic acid in an approximate ratio of 5:1, this could account both for the iodine number and for stearic acid as being the main hydrogenation product of the isolated fatty acid mixture.

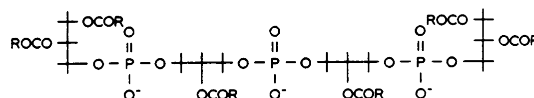

FIG. 1.—Concept of the structure of cardiolipin (Pangborn, 1947).

Results of partition chromatography (Faure and Coulon, 1948) and of column adsorption experiments (Rice and Osler, 1950; Rice, 1958) have thrown doubt on the identity of different cardiolipin preparations and have suggested the latter to be mixtures of similar compounds.

Analytical data obtained by Faure and Morelec-Coulon (1956, 1958) gave a molar ratio for glycerol:phosphorus of 3:2. Macfarlane and Gray (1957) and Gray and Macfarlane (1958), confirming these results, suggested for cardiolipin a diphosphatidylglycerol structure with a free median β-hydroxyl group and the phosphatidyl groups arbitrarily assigned to the $\alpha\gamma$-positions (Fig. 2).

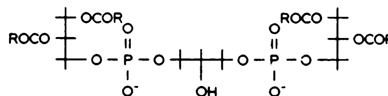

FIG. 2.—Present concept of the (diphosphatidylglycerol) structure of cardiolipin.

Evidence in favour of this structure was obtained by oxidative degradation with periodate of the polyglycerophosphate prepared by mild alkaline deacylation of cardiolipin (Macfarlane, 1958). Subsequently, the position of the fatty acids was ascertained by identification of the fragments obtained on hydrolysis of cardiolipin with hot acetic acid (Coulon-Morelec and Faure, 1958; Macfarlane and Wheeldon, 1959). Inasmuch as this hydrolysis occurs *via* the formation of a cyclic phosphoric diester, the presence of the free hydroxyl group was found to be essential (Coulon-Morelec, Faure, and Maréchal, 1960).

Considering various structures for cardiolipin, Macfarlane (1964) included the $\alpha\beta$-diphosphatidylglycerol structure. However, LeCocq and Ballou (1964) established its $\alpha\gamma$-configuration by showing the chromatographic identity between glycerodiphosphate obtained by degradation of cardiolipin and the synthetic $\alpha\gamma$-compound.

* Received for publication October 11, 1965.

† As an example, using the 4·18 factor as recommended by Pangborn (1955) for calculating the phosphorus content of the 3rd International Reference Preparation of Cardiolipin from the data given on the label, the 4·43 per cent. found is not within the required 4·00–4·30 range.

Chemical Structure and Serological Activity of Cardiolipin-related Compounds from Natural Sources Other than Ox Heart

Polyglycerophosphatide from ox liver appeared to be identical with cardiolipin from heart muscle both in composition and serological activity (Faure, Morelec-Coulon, Maréchal, and Leborgne, 1959); moreover, the chromatographic and chemical properties of their degradation products were shown to be similar (Macfarlane, 1961). For rat liver cardiolipin, Rose (1964) proposed a triphosphatidylglycerol structure (Fig. 3), postulating that the β-phosphatidyl group would be very labile to acid hydrolysis, giving the diphosphatidyl compound and phosphatidic acid. Such a structure would in fact be a positional isomer of the formula of Pangborn (1947).

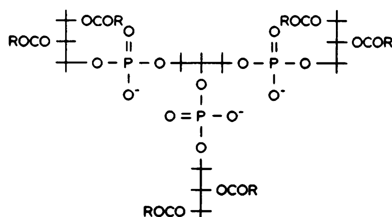

FIG. 3.—Triphosphatidylglycerol structure of rat liver cardiolipin (Rose, 1964).

Phosphatides from several plants were identified as diphosphatidylglycerols (Benson and Strickland, 1960). Sitolipin, isolated from wheat germs (Uroma and Louhivuori, 1951; Meinicke and Scheffel, 1954), is the only vegetable phosphatide that equals cardiolipin in serological activity and that has found limited application as an antigenic constituent in flocculation and complement-fixation tests for syphilis (Uroma and Tuomioja, 1951; Uroma and Tommila, 1951; Rein, Kelcec, and Rosenfield, 1951; Vogelsang, 1952). Phosphatidic acids from cabbage leaves, carrots, green-pea meal, and groundnuts have been reported to exhibit less serological activity with syphilitic serum than cardiolipin (Faure, 1949); this may be associated with their simpler monoglycerophosphatidic acid skeleton (Fig. 4). Hanahan and Chaikoff (1947, 1948) showed the phosphatidic acids of carrots and cabbage leaves to arise from enzymatic degradation of nitrogenous phosphatides. Whereas lecithin is completely inactive as an antigen, activity develops upon removal of the choline group by phospholipase D action. Thus, a free phosphoric acid group seems to be essential for serological activity (Mitra and Ghosh, 1963).

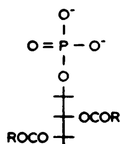

FIG. 4.—Monoglycerophosphatidic acid structure.

Serological Activity of Cardiolipin Degradation Products and of Synthetic Phosphatidic Acids

Faure and Coulon-Morelec (1963) found that a decrease in serological activity was always associated with chemical degradation of cardiolipin, for example, by reducing the appropriate number of glycerol and phosphoric acid groups. A complete loss of activity results from the removal of more than two of the four fatty acid chains present (see Fig. 2). Increasing the degree of saturation of the fatty acids decreased serological activity. The decrease during preservation of cardiolipin in alcoholic solution is mainly due to oxidation by air contact. Esterification of the free hydroxyl group with oleic or acetic acid reduced serological activity by factors of two and four respectively.

Inoue, Nojima, and Tomizawa (1965) synthesized palmitic acid containing cardiolipin analogues. Compared with the natural product, serological activity was found to be progressively decreased by substituting the median CHOH group by CH_2 and subsequently increasing the number of CH_2 groups of the central moiety to four or reducing this number to two. From these results, the distance between the two phosphoric acid groups (*c.* 7·5 Å in cardiolipin) was considered to be an important factor.

Allen and Tonks (1958) tested synthetic lauroyl, myristoyl, and oleoyl compounds of the α-monoglycero- and α-diglycerophosphatidic acid types (Figs 4 and 5). They found a minimal chain length of 14 C atoms in the fatty acid groups to be required for serological activity. In search of a synthetic substitute for cardiolipin, most promising results, both in the VDRL microflocculation test and in the Kolmer complement-fixation test, were obtained with monosodium β-dioleoyl-glycerophosphate, prepared by Foit and Schindler (1956). However, when compared with standard antigens, even in these cases qualification did not exceed "moderately reactive".

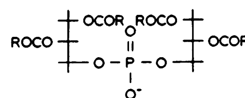

FIG. 5.—Diglycerophosphatidic acid structure.

Serological Activity of Synthetic Cardiolipin

Recently, de Haas and van Deenen (1965a) reported the synthesis of diphosphatidylglycerol, which contains equimolar amounts of stearic and oleic acids. Comparison of ox heart cardiolipin with the synthetic compound revealed no differences in chromatographical behaviour, infra-red absorption, melting point, or optical rotation. Mild alkaline hydrolysis of the natural and of the synthetic product yielded water-soluble phosphate esters indistinguishable by paper electrophoresis and chromatography. Both compounds were degraded in the same way by phospholipases A and C (de Haas and van Deenen, 1965b).

Solutions of the synthetic cardiolipin, natural lecithin, and cholesterol in dehydrated ethanol were mixed and constituted to give antigens with the following compositions: 0·0175 per cent. cardiolipin, 0·0875 per cent. lecithin, and 0·3 per cent. cholesterol for the Kolmer test, and 0·03 per cent. cardiolipin, 0·21 per cent. lecithin, and 0·9 per cent. cholesterol for the VDRL test. These antigens were tested in parallel with similar mixtures prepared with the same lecithin and cholesterol, but containing natural cardiolipin instead of the synthetic product.

The Kolmer complement-fixation test was carried out in its 1/5-volume modification employing two "exact units" of complement (Kolmer, 1942). In titrations with human syphilitic serum, both antigens showed an almost identical pattern with the usual prezone phenomenon (Table I). Despite the fact that both antigens exhibited an optimal activity in the 1:400 dilution in the present experiment with a high titred serum, it was decided to employ a 1:200 dilution in the comparative investigation because of the observed tendency of optimal antigen dilutions to decrease somewhat with serum titre*. 372 sera have been tested in the quantitative complement-fixation test, starting with undiluted serum (Table II).

TABLE I

PARALLEL TITRATION OF SYNTHETIC AND NATURAL CARDIOLIPIN-CONTAINING ANTIGENS WITH SYPHILITIC SERUM IN THE COMPLEMENT-FIXATION TEST

Serum Dilutions	Synthetic Cardiolipin-containing Antigen Dilutions					
	1:25	1:50	1:100	1:200	1:400	1:800
1:10	++++	++++	++++	++++	++++	++++
1:20	++++	++++	++++	++++	++++	++++
1:40	+	++	++++	++++	++++	++
1:80	—	—	—	—	+	—
Antigen Control	—	—	—	—	—	—
Serum Dilutions	**Natural Cardiolipin-containing Antigen Dilutions**					
	1:25	1:50	1:100	1:200	1:400	1:800
1:10	++++	++++	++++	++++	++++	++++
1:20	++++	++++	++++	++++	++++	++++
1:40	—	—	+	++++	++++	++
1:80	—	—	—	—	+	—
Antigen Control	—	—	—	—	—	—

Divergence in titre of the 167 sera that were reactive with both antigens is expressed in Table III. Results were similar to what might be expected when one antigen is tested in duplicate.

TABLE II

COMPARISON OF REACTIVITY BETWEEN SYNTHETIC AND NATURAL CARDIOLIPIN-CONTAINING ANTIGENS IN THE COMPLEMENT-FIXATION TEST

Natural	Synthetic		
	+	—	Totals
+	167	3 (*a*)	170
—	6 (*a*)	196	202
Totals	173	199	372

(*a*) Only partial inhibition of haemolysis in tube with undiluted serum.

TABLE III

COMPARISON OF TITRES OBTAINED WITH SYNTHETIC AND NATURAL CARDIOLIPIN-CONTAINING ANTIGENS IN THE COMPLEMENT-FIXATION TEST

Total	Synthetic = Natural	Synthetic > Natural	Synthetic < Natural
167	136	16 (*b*)	15 (*c*)

(*b*) Only one tube differences except in one case with a two tube difference.
(*c*) Only one tube differences.

In the qualitative VDRL microflocculation test, results obtained with the antigen containing synthetic cardiolipin compared favourably with those of standard antigen. The former gave even more clear-cut positive reactions without altering the specificity of the test.

For the first time a synthetic product is reported to be qualified as a substitute for natural cardiolipin in syphilis serology. From the standardization point of view of its defined chemical composition (*e.g.* phosphorus content, found: 4·08 and 4·13 per cent.; calculated: 4·11 per cent.†), it contrasts favourably with the variation inherent in the natural compound. It should be noted that in ox heart cardiolipin the fatty acids consist of about 80 per cent. linoleic acid, whereas the synthetic product contains equimolar amounts of stearic and oleic acids. The results obtained in our experiments indicate that the degree of unsaturation of the fatty acid chains apparently is not of primary importance for serological activity. The reduction in the number of double bonds is likely to decrease the rate of oxidation and can be expected to have a favourable effect on keeping qualities. Even if reasons of economy were to prevent the general application of synthetic cardiolipin (preferably in combination with synthetic lecithin) in the sero-diagnosis of syphilis, it would be worthwhile to consider its use as an international standard.

* Unpublished observations.
† G. H. de Haas, personal communication.

Summary

(1) A review is presented of the literature on the chemical structure of cardiolipin and the serological activity of similar synthetic compounds.

(2) Results obtained in the Kolmer complement-fixation test and VDRL microflocculation test, suggest that synthetic diphosphatidylglycerol may be used as a substitute for natural cardiolipin.

The author wishes to thank Prof. Dr L. L. M. van Deenen and Dr G. H. de Haas (Biochemical Dept of the Organic Laboratory of the State University of Utrecht) for the generous supply of their synthetic diphosphatidylglycerol. He is indebted to Miss J. H. de Jong for carrying out the serological experiments and to Dr J. N. Miller (Los Angeles, USA) for reading the manuscript.

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La structure chimique et l'activité sérologique du cardiolipide naturel et synthétique et des composés analogues

Résumé

(1) La structure chimique du cardiolipide et l'activité sérologique des composés analogues décrites dans la littérature sont passés en revue.

(2) Le diphosphatidylglycérol synthétique peut remplacer le cardiolipide naturel dans la réaction de fixation du complément (technique de Kolmer) et dans la réaction microscopique de VDRL.