

Chemical Structure and Serological Activity of Synthetic and Natural Cardiolipin

CARDIOLIPIN was first isolated by Pangborn¹ from ox-heart and this compound proved to be the essential antigen in the serodiagnosis of syphilis. Based on hydrolysis investigations, Macfarlane *et al.*^{2,3} and Faure and Morelec-Coulon^{4,5} arrived at the conclusion that cardiolipin has a diphosphatidyl glycerol (I) structure. Recently, LeCocq and Ballou⁶ confirmed this view, Rose⁷ favoured a structure isomeric to that originally proposed by Pangborn¹. Conclusive evidence about the structure of cardiolipin has now been obtained by means of chemical synthesis.

We have succeeded in the preparation of diphosphatidyl glycerol (I) by a condensation reaction between silver α -oleoyl- β -oleoyl- γ -glycerobenzyl phosphate and α , γ -dibromoglycerol- β -*t*-butylether followed by removal of the protecting benzyl and *t*-butylether functions⁸. Comparison of ox-heart cardiolipin with the synthesized compound revealed no differences in chromatographical behaviour, infra-red absorption, melting point and optical rotation (natural cardiolipin $[\alpha]_D^{20} + 5.5^\circ$; synthesized diphosphatidyl glycerol $[\alpha]_D^{20} + 5.8^\circ$, both in chloroform *d*. 10). Mild alkaline hydrolysis of the natural cardiolipin and the synthetic material yielded water-soluble phosphate esters indistinguishable by paper electrophoresis and chromatography. Final proof for the identity of natural cardiolipin and synthetic diphosphatidyl glycerol was obtained by enzymatic hydrolysis with the sphingolipases A (*EC* 3.1.1.4) and C (*EC* 3.1.4.3). Short-term incubation of both phospholipids with *Crotalus maurus* venom (in the presence of stearylphosphoryl line which reduces the negative charge of the lipidelles) resulted in the formation of two lyso-derivatives giving one and two β -fatty acids respectively. After prolonged incubation both phospholipids were quantitatively converted into one slow-moving lysophospholipid containing half the amount of fatty acids originally present¹⁰.

In accordance with the findings of Ottolenghi¹¹ the action of phospholipase C appeared to be catalysed by cations. A short incubation of both substrates with the enzyme of *Bacillus cereus* yielded a 1,2-diglyceride and a sphingolipid which was found to be chromatographically identical with chemically synthesized phosphatidyl glycerophosphate¹². Prolonged action of this enzyme converted both the synthetic and isolated cardiolipin completely into 1,3-diphosphoglycerol (not contaminated with the 1,2-isomer) and 1,2-diglyceride. These findings unequivocally demonstrate that cardiolipin from ox-heart is identical to 1,3-diphosphatidyl glycerol. A great number of synthetic acidic phosphatides have been investigated as possible substitutes for cardiolipin in the serological tests for syphilis^{13,14}; none of these synthetic samples revealed an activity which equalled that of ox-heart cardiolipin. The serological activity of our synthetic product was compared with that of ox-heart cardiolipin by Dr. J. H. de Bruijn of the National Institute of Public

Health (Utrecht). At present more than one hundred sera have been investigated. In the Kolmer complement fixation test the reactivity of the synthesized diphosphatidyl glycerol appeared to be indistinguishable from those of ox-heart cardiolipin. In the VDRL slide test the synthesized compound gave even more clear-cut positive results without altering the specificity of the reaction. It should be noted that ox-heart cardiolipin consists of about 80 per cent of linoleic acid in contrast to our synthetic product, which contains equimolar amounts of stearic and oleic acid. The results obtained in the serological assays indicate that the degree of unsaturation of the fatty acids apparently is not of primary importance for the activity of cardiolipin in this reaction. With regard to the relation between structure and activity of acidic phospholipids in the serological assays for diagnosis of syphilis, it will be of interest to evaluate the significance of the zeta-potential of the micelles of the lipid antigen.

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'Isoallergens' from Rye Grass Pollen

SEVERAL methods for the purification of allergens from pollens and from other vegetable and animal sources have been reported¹⁻⁹, but recently, by utilizing highly selective chromatographic procedures, it has been possible to obtain allergens of much higher purity than hitherto described^{10,11}. At least two groups of allergens have been found in rye pollen, a major group, active in the majority of sensitive patients, and a minor group, active in only a small proportion. It seems likely that such groups also exist in other grass and ragweed pollens causing hay-fever.

The method of isolating rye pollen allergens, described in detail elsewhere¹⁰, was briefly as follows. Ether-defatted rye grass pollen was extracted with 0.001 M NH_4HCO_3 at pH 8 and the clarified extract was dialysed against the same solvent. The allergenic non-dialysable material was then concentrated by freeze-drying (yield: 6 g/100 g defatted pollen). This process has repeatedly been shown to yield virtually all the allergenic substances^{1,3,10,12}, assayed by quantitative skin testing on allergic volunteers¹³. Allergenic components were then isolated by fractionation of dialysed extracts successively on columns of DEAE-cellulose and 'Sephadex G75'. Homogeneity of the fractions was assessed by starch-gel electrophoresis (Fig. 1), ultracentrifugation, behaviour on 'Sephadex G75', N-terminal amino-acid analysis and immuno-diffusion against rabbit anti-rye pollen sera. Their skin activity, relative to whole-pollen extract, was shown to increase at each stage of purification of the allergenic fractions.

Two allergens, α and β , were each homogeneous to the foregoing criteria and by gel electrophoresis appeared to be immunologically identical.

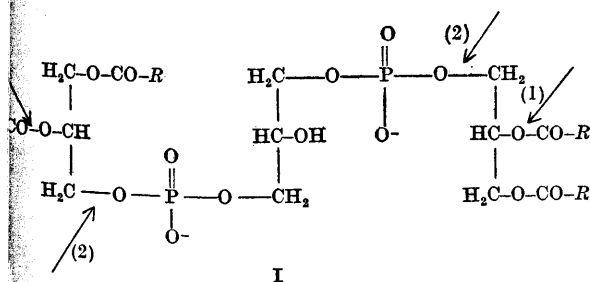


Fig. 1. Structural formula of diphosphatidyl glycerol (cardiolipin). Arrows (1) and (2) indicate the sites of attack of phospholipase A and phospholipase C respectively.

10). In many physical and chemical properties (including molecular weight, amino-acid analysis, carbohydrate compositions and peptide fingerprints) they were almost identical^{10,14}, but the β -allergen migrated more rapidly than the α in starch-gel electrophoresis at pH 8.5 (Fig. 1 and ref. 10). Certain cruder fractions, for example, I (ref. 14), contained, in addition to α and β , small quantities of two closely related, antigenically identical components, possessing mobilities in gel electrophoresis which were less than and greater than both α and β , respectively (Fig. 1). The foregoing allergenic fractions and the purified allergens (α and β) were highly skin-active in all 16 rye-sensitive individuals so far tested: their specific activities, S , relative to the whole non-dialysed extract, were experimentally indistinguishable (average $S = 130$ by graphical methods^{13,10}). Fractions α and β and the related components have been termed major allergens.

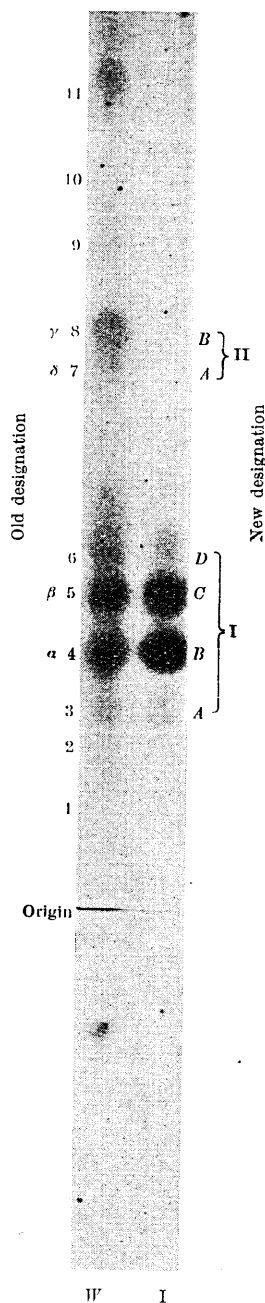


Fig. 1. Starch-gel electrophoretic patterns in borate buffer at pH 8.5 for a dialysed whole-rye pollen extract, IV, and the major allergenic fraction I. (Protein-staining by amido-black 10B)

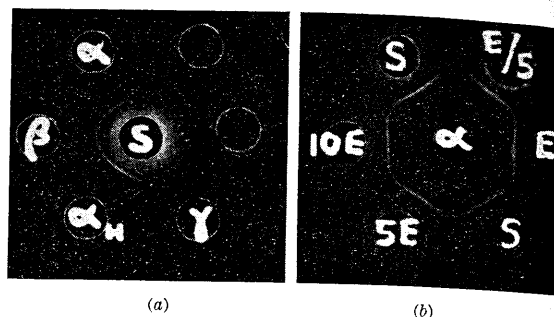


Fig. 2. Immunodiffusion patterns. *a*, For purified rye pollen allergens against rabbit antiserum S . Fraction α_H was heated for 10 min at 100°C in physiological saline at pH 7.3; *b*, for tests of possible inhibition of precipitin line formation between the major rye allergen, α (I-B), and rabbit antiserum, S . 10E, SE, etc., refer to the number of equivalents of γ (II-B) mixed with the antiserum prior to diffusion

Their molecular weight was shown by physical method to be 32,000–34,000 in fair accord with that (31,500) suggested from amino-acid analytical results. The very close similarities in the physical properties, amino-acid analyses and tryptic and chymotryptic fingerprints of α and β have led to the suggestion that α and β may differ solely in their amide content¹⁰.

Two further allergenic fractions, γ and δ , of molecular weight about 10,000 were skin-active in a small proportion of rye-sensitive individuals (with $S = 600$) and are considered to be members of a minor group. Fractions γ and δ possessed the same rate of migration on 'Sephadex G75'; neither could be shown to elicit the formation of the precipitating antibody in the rabbit, but they were distinguishable by their different rates of migration in starch-gel electrophoresis at pH 8.5 (Fig. 1).

According to gel-immunodiffusion analysis, fraction γ failed to inhibit precipitin line formation between α and rabbit anti-rye serum, when up to 20 molar equivalents were mixed with the serum prior to diffusion (Fig. 2). This result established the non-identity of antigenic determinants for the major and minor fractions. An indication of the non-identity of allergenic determinants was suggested by the fact that the minor group of allergens is active only in a small proportion of the individuals who are sensitive to the major group. This was further substantiated when it was shown that a large excess of fraction γ , mixed with allergen β , failed to inhibit skin reactions in those individuals sensitive only to the major allergens.

A series of experiments was performed in an attempt to locate the origin of the biological activity of the allergen molecules. Total tryptic, chymotryptic and peptic digests of α all failed to inhibit the formation of a precipitin line between α (or β) and rabbit antisera: these digests were also completely non-allergenic. A glycopeptide fragment, isolated after extensive enzymatic digestion of α (ref. 10), behaved in the same manner. However, certain partial peptic and chymotryptic digests did inhibit precipitation though possibly due to unaltered allergens. These results, together with others utilizing chemically modified allergens (to be published), indicate that the tertiary structure of certain parts of the major allergens is probably essential for full antigenic and allergenic activity.

Preliminary results using timothy grass pollen revealed that a prominent allergenic fraction possessed similar electrophoretic and chromatographic properties to the major rye allergens. Malley *et al.*⁵ also observed allergenic activity in a fraction of molecular weight 9,000–10,000 (cf. rye allergens γ and δ). However, a more striking comparison may be made between the rye allergens and those of ragweed pollen isolated by King and Norman¹. Allergenic activity occurred in two different types of fraction as in the cases of rye and timothy pollen. A major group of four allergens, with molecular weights of abo

and equal activity in all ragweed-sensitive individuals gave a 4-band starch-gel electrophoretic pattern similar to that of rye¹⁶. An allergenic fraction of molecular weight ($\approx 10,000$), which was not fully prized but which demonstrated skin-activity only in ragweed-sensitive individuals, also occurred. Further, the ragweed allergens possessed quite different ability to enzymatic hydrolysis; and with certain differences in physical properties, it is clear that the ragweed and rye allergen molecules differ significantly. A common feature seems to be the pattern in which allergenic activity is distributed among various materials derived from the pollens. Since, in each case, groups of more than single allergens occur, it seems appropriate to refer to the members of a group as 'isoallergens' by analogy with 'isoenzymes'¹⁷, and convenient to introduce a nomenclature whereby corresponding isoallergenic groups, as individual members for different pollens, could be compared. Thus the major and minor groups could be designated I and II respectively, and individual allergens listed alphabetically according to apparent mobility on starch-gel electrophoresis at alkaline pH (cf. King¹⁶). Hence rye allergens comprising bands 3, 4(α), and 6 on starch-gel (Fig. 1) are termed I-A, B, C, D, and King's ragweed allergens IV-A, B, C, D and I-A, B, C, D. Rye allergens γ and δ now become II-A, respectively.

Worth¹⁸ has recently suggested that the carbohydrate moieties of allergenic substances are probably important for allergenic activity. Substantial evidence in support of such a hypothesis being applicable to pollen allergens is now accumulated. Augustin¹⁹ found that several hydrolases did not affect the allergenic activities of rye and cocksfoot pollen extracts. Furthermore, it is strongly indicated that neither alder nor ragweed allergens contain carbohydrate^{2,11}. The failure of an epitope fragment from allergen α to inhibit the antibody reaction between α and rabbit anti-rye serum adds weight to the view that a carbohydrate is not important allergenically. On the other hand, some alteration and inactivation of this fragment on hydrolysis cannot yet be ruled out.

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Isolation and Partial Characterization of a Pink Pigment from Normal Human Plasma

DURING our work on the very soluble constituents of normal human plasma a pink pigment was discovered.

Normal human plasma was fractionated according to Cohn method 6 (ref. 1). From the supernatant solution of fraction V obtained by this technique, fraction VI² was rendered insoluble at pH 5.8 and -5° by addition of 1 M ZnAc_2 to give a final concentration of 0.06 M. The resulting precipitate was dissolved with pre-cooled neutralized ethylenediamine tetraacetate and dialysed against cold distilled water. A preliminary separation of the components of fraction VI was achieved by chromatography on a DEAE-cellulose column at pH 5. The major part of the proteins including albumin, α_1 -acid glycoprotein³ and the 3S α_2 -glycoproteins² was retained, whereas the effluent contained the relatively basic compounds including certain basic peptides³ and the γ -globulins of low molecular weight⁴. The effluent was applied afterwards to a CM-cellulose column at pH 6. A pink zone was noted near the upper end of the column. Fractional elution utilizing an ionic strength gradient led to the release first of the 3S γ_1 - and afterwards of low-molecular-weight γ_2 -globulins. Thereafter the pink zone was displaced at an ionic strength of 0.6. The solutions obtained were separately dialysed and lyophilized.

The resulting pink preparation of which the weight was approximately 0.2 mg/l. of pooled outdated normal human plasma appeared heterogeneous on paper electrophoretic analysis at pH 8.6 (Fig. 1). The unstained electropherograms showed a pink band which had moved toward the cathode. After staining with amido black, four zones were noted with electrophoretic mobilities corresponding to the α_2 -, β_1 - and γ_2 -globulins and to the basic compound B_2 (ref. 4), respectively, the latter forming the major band. The pink pigment having the highest cathodic electrophoretic mobility was clearly separated from the basic compound but did not stain with amido black. The absorption spectrum of this chromogen (Fig. 2) revealed a major maximum at 407 m μ and two minor maxima at 516 and 540 m μ . As expected, the pink compound does not exhibit vitamin B₁₂ activity, as vitamin B₁₂ is known to undergo changes very rapidly on exposure to light⁵. Further investigations pertaining

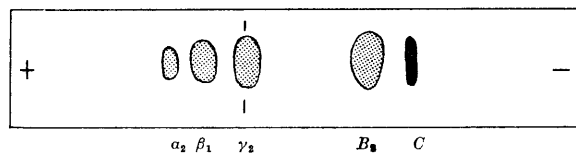


Fig. 1. Paper electrophoretic analysis at pH 8.6 of the human plasma fraction containing a pink chromogen (C). The position of the components demonstrable with amido black 10B, α_2 -, β_1 - and γ_2 -globulins and the basic compound B_2 , are indicated by dotted areas

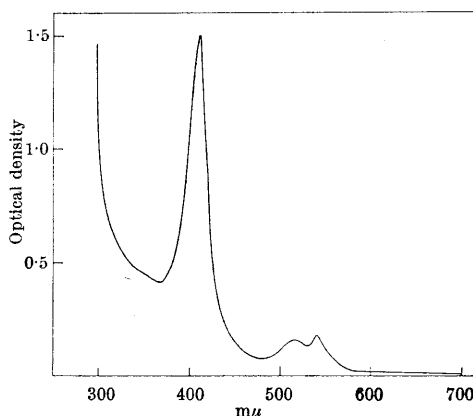


Fig. 2. Spectrum of the partially purified pink chromogen of normal human plasma