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A Surface-Chemical Study of a Hapten-Antibody Reaction: The Reaction of Wassermann Antibody

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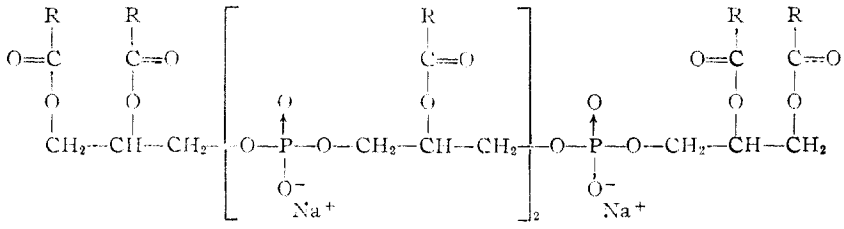
The hapten-antibody system for which the antibody is human Wassermann antibody and cardiolipin the hapten, has been investigated by the monolayer technique. The experimental study reported here has centered about the demonstration that the specific reaction can be detected by this method, due to the different *p*H dependence of the rate of interaction of a hapten monolayer spread at an air-water interface with its substrate containing homologous antibody or normal human γ-globulin. The results are interpreted to mean that a monolayer of pure cardiolipin is not haptenic toward Wassermann antibody but that mixed films of cardiolipin-cholesterol and cardiolipin-lecithin do undergo specific reactions. The possibility that other polar molecules, when mixed with cardiolipin, also complete the haptenic requirements of Wassermann antibody, is discussed briefly.

The interaction of built-up molecular layers of antigens with their homologous antibodies has been studied by a number of investigators.[2-7] In the earlier studies, one of the foci of interest was whether a protein antigen, spread at an air-water interface before transfer to a metal or glass plate, retained its ability to react specifically with its homologous antibody. When the retention of specific reactivity in built-up films was demonstrated, it was considered that the antigen specific group in these cases is so situated on the molecular surface and sufficiently small, that it is not disrupted during the process of unfolding. In view of this, the failure of serum globulin, spread at an air-water interface, to react with its homologous rabbit antiserum injected into the substrate[8] is surprising if not contradictory. In recent years the transferred film technique has formed the experimental basis for the controversy over long-range forces[9-16] and little use has been made of it in the study of chemical factors determining the specificity of antigen-antibody reactions.

However, it appears that the technique of monolayer substrate interactions should be well suited to the study of those systems in which one component may be spread at an air-water interface without denaturation or loss of specificity.[17] In particular if antigen-antibody reactions could be carried out on a surface balance, many details of the reaction could be explored that are not open to investigation by the transferred film technique.

Considering the desirability of having the antigen or hapten spread reproducibly at the interface, it appears that the system of the hapten and antibody used to detect the presence of syphilis offers attractive possibilities.[19] All the current serological flocculation tests[20] depend on the ability of the Wassermann antibody in the sera of syphilitic subjects to react specifically with unstable lipid suspensions, causing rapid coagulation, while negative sera disperse these suspensions more finely. The "antigen" suspensions in common use consist of alcoholic extracts of beef-heart muscle, containing phosphatides and lipids, with cholesterol added. It was thought at one time that cholesterol and lecithin were the specific ingredients of these mixtures but between 1942 and 1945 Pangborn[21-24] isolated a phospholipid, cardiolipin, and identified its constituents to be 4 molecules of glycerol, 3 of phosphoric acid and 6 molecules of unsaturated fatty acid most probably in the ratio of 5 oleic to 1 linoleic. The structure which she proposed accounted for the properties of its monolayer as studied by Doty and Schulman[19] and Glazer[25] and is



Since then, suspension of pure cardiolipin, cholesterol and lecithin in 0.85% NaCl have been shown to be very suitable for use as "antigens" in the specific reaction with Wassermann antibody.[26-28] This

(1) Predoctoral Research Fellow of the National Institutes of Health, United States Public Health Service for the year 1950-1951.

(2) L. A. Chambers, J. B. Bateman and H. E. Calkins, *J. Immunol.*, **40**, 483 (1940).

(3) L. Fourt, P. O. Fourt and W. D. Harkins, *J. Biol. Chem.*, **132**, 11 (1940).

(4) I. Langmuir and V. J. Schaeffer, *Chem. Revs.*, **24**, 181 (1939).

(5) E. F. Porter and A. M. Pappenheimer, *J. Exp. Med.*, **76**, 437 (1937).

(6) A. Rothen and K. Landsteiner, *ibid.*, **76**, 437 (1942).

(7) A. Rothen, "Adv. Protein Chemistry," Vol. III, New York, N. Y., 1947, p. 123.

(8) J. E. Danielli, F. Danielli and J. R. Marrack, *J. Brit. Soc. Exp. Pathology*, **19**, 393 (1938).

(9) G. Oster, *J. Gen. Physiol.*, **33**, 445 (1950).

(10) A. Rothen, *Science*, **102**, 446 (1945).

(11) A. Rothen, *J. Biol. Chem.*, **168**, 75 (1947).

(12) A. Rothen, *Helv. Chim. Acta*, **33**, 834 (1950).

(13) A. Rothen, *Science*, **112**, 330 (1950).

(14) S. J. Singer, *J. Biol. Chem.*, **182**, 189 (1950).

(15) H. J. Trurnit, *Science*, **111**, 1 (1950).

(16) H. J. Trurnit, *ibid.*, **112**, 329 (1950).

(17) The technique has been used to follow a number of other reactions. For a review of the results and references to the original literature, see ref. 18.

(18) E. K. Rideal, *J. Chem. Soc.*, 423 (1945).

(19) P. M. Doty and J. H. Schulman, *Disc. Far. Soc.*, No. 6, 21 (1949).

(20) *Venereal Disease Information Supplement*, **22**, Supp. No. 14, Fed. Security Agency (1949).

(21) M. Pangborn, *J. Biol. Chem.*, **153**, 343 (1944).

(22) M. Pangborn, *ibid.*, **157**, 691 (1945).

(23) M. Pangborn, *ibid.*, **161**, 71 (1945).

(24) M. Pangborn, *ibid.*, **168**, 351 (1947).

(25) J. Glazer, *Disc. Far. Soc.*, No. 6, 39 (1949).

(26) R. Brown, *J. Immunol.*, **53**, 171 (1946).

(27) B. S. Kline, *Am. J. Clin. Path.*, 48 (1946).

(28) C. R. Reid and H. N. Bossak, *Am. J. Syphilis, Gonorrhea and Ven. Dis.*, **30**, 40 (1946).

hapten–antibody system, one reactant of which may be spread at an air–water interface without permanent structural alteration, was thus thought to be well suited to an investigation of the potentialities of the method in the study of immunochemical reactions.

The experimental work reported here has centered about demonstration that the reaction can be detected by the use of the monolayer technique and that this can then be employed to determine the nature of the haptenic group in the hapten–antibody reaction in which the antibody is human Wassermann antibody. In addition we have explored in a limited way the role of the other non-haptenic compounds used in the serological tests for syphilis. As a basis for this work it was first necessary to study the interaction of normal human γ-globulin with monolayers of cardiolipin[19,29,30] and eventually with mixed films, in order to establish a basis for comparison of the specific and non-specific surface reactions.

Materials, Apparatus and Experimental Method

Materials.—Several samples of cardiolipin prepared by the method of Pangborn were kindly given to us by the Lederle Laboratories Division of the American Cyanamid Co., Pearl River, New York, in the form of 0.5 to 0.8% solutions in ethyl alcohol. For the purpose of spreading in monolayers, these solutions were diluted 1:10 with redistilled CCl_4. Alcohol solutions of lecithin were also obtained from the Lederle Co.

The Wassermann antibody was made by the method of Davis, Moore, Kabat and Harris[31] from human sera and citrated blood. The human γ-globulin was fraction II-1 produced by the procedure developed by Cohn, *et al.*[32] It has an isoelectric range of 6.5 to 7.5 in 0.02 *M* NaCl, and is the fraction in which immune proteins are found.

For flocculation tests on sera and purified antibody, the procedure of the "quantitative" Kahn test was used.[33] The standard Kahn antigen was obtained from Difco Laboratories, Detroit.

Buffers were made from reagent grade citric acid and disodium phosphate in redistilled water and treated with activated charcoal to remove surface-active materials. They were then centrifuged free of charcoal before use.

Apparatus.—The surface balance was a small instrument made at the Department of Colloid Science, Cambridge University, capable of distinguishing surface pressures of 0.05 to 0.1 dyne/cm. It was equipped with a single torsion wire and a mica boom, which was attached to the sides of the glass trough by two short vaseline-treated silk threads.[34] The film of surface active material was confined between the boom and a paraffined glass slide. The apparatus was enclosed in an air thermostat, allowing the temperature in any single experiment to be controlled within 0.5°; the position of the barrier and the torsion on the wire could be adjusted from outside. The balance was used as a zero point instrument, that is, as the surface pressure changed, the torsion on one end of the wire was adjusted to keep the boom always in the same position. The glass trough was 14 × 32 cm. and sufficiently deep to hold 550 ml. of buffer. In the penetration experiments, in order to maintain the substrate composition constant throughout, it was continuously stirred by means of a five-pronged pipet with its orifices distributed below the surface, into which 30–40 ml. of solution was sucked 4–5 times every 5 minutes, and allowed to flow back under their own power.

Solutions of surface active materials were added from a calibrated microsyringe, similar to the type sold in England under the trade-mark of "Agla," from which 0.035 ml. could be delivered with a reproducibility of better than 1%. The concentration of γ-globulin and antibody was determined spectrophotometrically using the value of 14.5 for the extinction coefficient of human γ-globulin at 279 mμ.[37] We have at no time had sufficient human Wassermann antibody to be able to determine whether its extinction coefficient is that of normal human γ-globulin. A systematic error may therefore have been introduced. The reasons for its lack of significance will be discussed below.

Performance of Penetration Experiments.—In studying reactions between monolayers and substrates, it is generally desirable to measure equilibrium properties, *e.g.*, the total increase in surface area at constant pressure. Preliminary experiments on this system showed, however, that equilibrium was not attained even in several hours following the injection of antibody into the substrate. It was therefore necessary to explore the possibility of using the initial rate of increase of surface pressure at constant area or the initial rate of increase of surface area at constant pressure as a means of characterizing the reaction. The experimental procedure which was decided upon is the following:

The cardiolipin or other lipid solution is spread on the water surface with the microsyringe. A certain amount of time is allowed to elapse between the spreading and compression of the film to ensure complete unfolding and temperature equilibrium. The film is then compressed to the surface pressure at which the penetration experiment is to be performed. The compression is carried out stepwise over a period of 30 minutes. The constancy of surface pressure with mixing is then tested over a period of 15 to 20 minutes. (A decrease of surface pressure during this process is due to slow leakage of the film past the confining threads or barrier, while constantly increasing surface pressures are the result of contamination.) If changes of area are less than 1% per hour, the experiment proceeds. If they are between 1 and 5%, they are followed over a longer period of time and the final result corrected for this error. If greater than 5%, the experiment is started again. (One exception was made to this last rule; Fig. 5 series F, *p*H 3.25.)

The addition of antibody or γ-globulin to the substrate is made as follows. A volume of protein solution giving the required amount of material is mixed with 10 ml. of buffer withdrawn from the substrate with a bent pipet. Ten ml. of the resulting solution is reinjected under the monolayer, and the substrate mixed for three minutes before readings are taken. The zero of time is arbitrarily taken as the time when the addition of protein to the substrate is complete. The change of area of the monolayer at constant surface pressure is then followed. The substrate is mixed by sucking material into the mixing pipet 4–5 times every 5 minutes. (At this rate of mixing, the reaction rate is not very sensitive to the mixing process, permitting this relatively crude but simple procedure to be used.) The area of the surface behind the mica boom is kept free of contaminating material by sweeping with a coated barrier every 10 minutes. Every 20 minutes the material so collected is sucked off with a fine-tipped aspirator. However, as a consequence of this slight removal of subtrate and other factors such as evaporation, the zero position of the mica boom is changed. Keeping it at the same position throughout the experiment results in the surface pressure being 0.3 ± 0.2 dyne/cm. lower at the end of the experiment than at the beginning.

Results

A. Determining the Experimental Conditions.—It was, as we have said, necessary to investigate the non-specific protein substrate–charged monolayer interaction as a prelude to the comparison and contrast of the specific and charge-mediated surface reactions. These experiments showed that the following factors influence the interaction of γ-globulin with the lipid monolayer: the surface pres-

(29) R. Matalon and J. H. Schulman, *Disc. Far. Soc.*, No. 6, 27 (1949).

(30) J. H. Schulman, *Biochem. J.*, **39**, LIV (1945).

(31) B. D. Davis, D. H. Moore, E. A. Kabat and A. Harris, *J. Immunol.*, **50**, 1 (1945).

(32) E. J. Cohn, *et al.*, THIS JOURNAL, **72**, 465 (1950).

(33) R. L. Kahn, "The Kahn Test," Williams and Wilkins Co., Baltimore, Md., 1928.

(34) For a description of similar instruments, see ref. 35 and 36.

(35) N. K. Adam, "The Physics and Chemistry of Surfaces," 3rd Ed., London, 1941.

(36) A. E. Alexander and P. Johnson, "Colloid Science," Vol. I, London, 1949, p. 491.

(37) F. N. Gurd, Thesis, Harvard University, 1948.

sure of the film, the ionic strength of the buffer, the concentration of protein in the substrate and the *p*H.

Considering first the influence of the surface pressure, it is usually recognized that the collapse pressure of protein films is about 15 dynes/cm.[38] If the surface film is kept above that surface pressure, spontaneous unfolding of the protein molecules at the interface is prevented.[39] For this reason it was originally hoped that by employing surface pressures of 18 dynes/cm. or higher, the charge-mediated and specific interactions could be studied by equilibrium methods. However, we found that even at fairly high substrate concentrations of protein (9 mg./l.) the interaction of globulin with cardiolipin at 18.5 dynes/cm. does not come to equilibrium within the time feasible for surface experiments. Figure 1 and curve 1 of Fig. 2 show that the monolayer at a constant surface pressure of 18.5 dynes/cm. expands continuously. Indeed at the highest concentration studied, a constant rate of extension of the film is observed after a certain initial period. This is the case irrespective of whether the substrate is mixed continuously or not (Fig. 2, curves 2 and 3), and at a surface pressure as high as 21.6 dynes/cm. which is 40% above the collapse pressure of the protein film. This behavior suggests that the cardiolipin monolayer is highly compressible due to its poor packing at the interface and that such a film may have many "holes" into which the protein can penetrate and unfold.

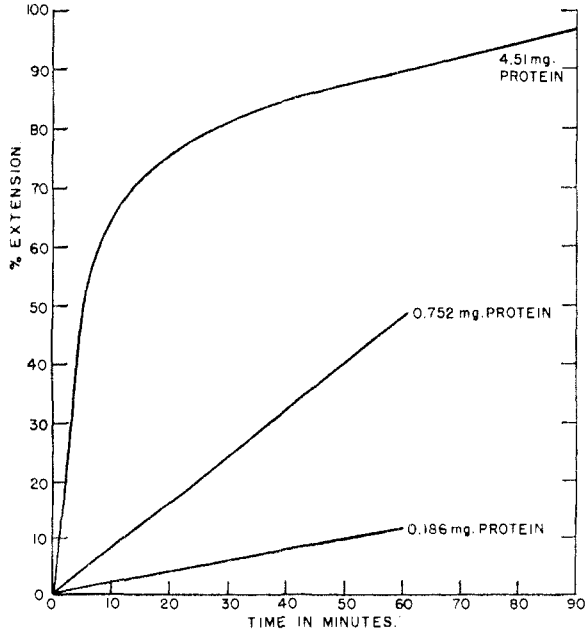


Fig. 1.—Influence of γ-globulin concentration on its rate of penetration into a cardiolipin monolayer at 18.5 dynes/cm. surface pressure. The quantities of protein refer to 550 ml. substrate solution (*p*H of substrate is 4.11; substrate not mixed during experiment; volume of substrate is 550 ml.)

(38) H. B. Bull, "Advances in Protein Chemistry," Vol. III, Academic Press, New York, N. Y., 1947, p. 95.

(39) J. H. Schulman and E. K. Rideal, *Proc. Roy. Soc. (London)*, **B122**, 29 (1937).

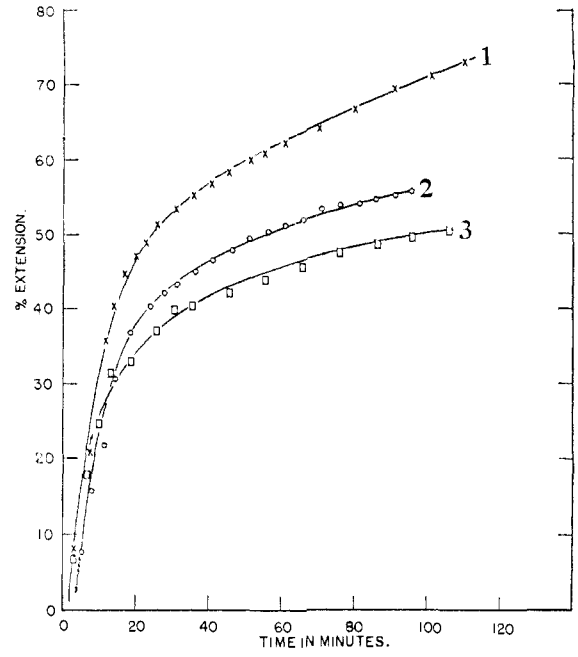


Fig. 2.—Influence of surface pressure and mixing of substrate on the rate of penetration of γ-globulin into a cardiolipin monolayer. Experimental conditions: Protein concentration 3.58 mg. in 550 ml.: curve 1, π = 18.5 dynes/cm., no mixing; curve 2, π = 21.6 dynes/cm., continuous mixing; curve 3, π = 21.6 dymes/cm., no mixing.

The influence of the substrate ionic strength on the protein–monolayer interaction is illustrated by the experimental results on the penetration of γ-globulin into a mixed film of cardiolipin and cholesterol (1:1 on a molecular basis). In Fig. 3 the rate of penetration of protein into the charged monolayer is plotted as a function of *p*H for two series of buffers. The ionic strength is not constant within each series of buffers but at any *p*H the buffer responsible for the point on curve 1 is approximately twice as concentrated as the buffer in the series giving rise to curve 2. (The composition of the buffers is tabulated in the note in Table I.) There appears to be a shift of the whole *p*H dependence curve to lower *p*H's when the buffer concentration is increased, suggesting that this effect is due to the binding of negative ions by the protein, resulting in a lowering of its isoelectric point.

In the absence of equilibrium, the experimental task resolves itself into a determination of relative rates of reaction of the antibody and γ-globulin at the same concentration with various monolayers over a *p*H range. The fact that only comparative rates have been sought has permitted the inclusion of an improvement in the method of performing the experiments, namely, continuous mixing of the substrate. This has had the twin beneficial effects of increasing the rate of penetration for a given concentration of protein, and reducing the experimental error. The choice of protein concentration is made with an eye both to economy of material and ease of characterizing the experimental data. At higher concentrations of protein (7–9 mg./l.) the extension of the monolayer is initially very rapid, and after a period of 20 to 40 minutes becomes much more gradual and almost

TABLE I

SUMMARY OF EXPERIMENTAL CONDITIONS OF THE EXPERIMENTS WHOSE RESULTS ARE SHOWN IN FIGS. 3, 4, 5 AND 6

Buffer I is the citrate–phosphate buffer,[40] at half the concentration; buffer II is buffer I treated with 5 g./l. of an activated carbon which also absorbs some citrate; buffer III is the buffer[40] after treatment with 5 g./l. of activated charcoal[a]; W. ab. = Wassermann antibody; γ-g. = human γ-globulin.

Experiment series	A	B	C	D	E	F	G
Monolayer	Cardiolipin		Cardiolipin–Cholesterol			Cardiolipin–Lecithin	
Protein substrate	W. ab.	γ-g.	W. ab.	γ-g.	γ-g.	W. ab.	γ-g.
Surface pressure, dynes/cm.	18.5			18.5		18.5	
Total protein in 550 ml., mg.	0.351	0.351	0.360	0.360	0.360	0.360	0.360
Temperature, °C.	29.5 ± 1	22.8 ± 0.8	25.3 ± 0.3	25.0 ± 0.5	24.7 ± 0.7	25.0 ± 0.7	24.9 ± 0.6
*p*H range	4–7	4–7	3–7	3–7	3–7	3–7	3–7
Buffer	I	I	II	III	II	II	II

[a] The buffer concentrations of series II and III (after charcoal treatment) are:

Buffer II			Buffer III		
Na_2HPO_4, *M*	Citric acid, *M*	*p*H	Na_2HPO_4, *M*	Citric acid, *M*	*p*H
0.0206	0.0350	3.27 ± 0.03	0.0411	0.0795	3.12 ± 0.04
.0362	.0270	4.28 ± .03	.0771	.0615	4.03 ± .04
.0443	.0256	4.75 ± .03	.0882	.0559	4.56 ± .04
.0518	.0233	5.31 ± .03	.103	.0485	5.11 ± .04
.0636	.0179	6.24 ± .03	.1263	.0369	6.08 ± .04
.0827	.00885	7.09 ± .03			

linear with time (Fig. 1, Fig. 2 curve 1). However, protein concentrations of 0.6 to 0.8 mg./l. (0.3 to 0.4 mg. in 550 ml.) give a linear extension of the monolayer during the entire 60-minute period of measurement (Fig. 1). The lower concentration of protein, approximately 0.35 mg. in 550 ml., was therefore chosen for all the later experiments. In all these experiments the expansion of the surface film at constant surface pressure was linear or almost linear with time. For this reason only the per cent. extension of the monolayer per hour at constant surface pressure is plotted in Figs. 3, 4, 5 and 6.

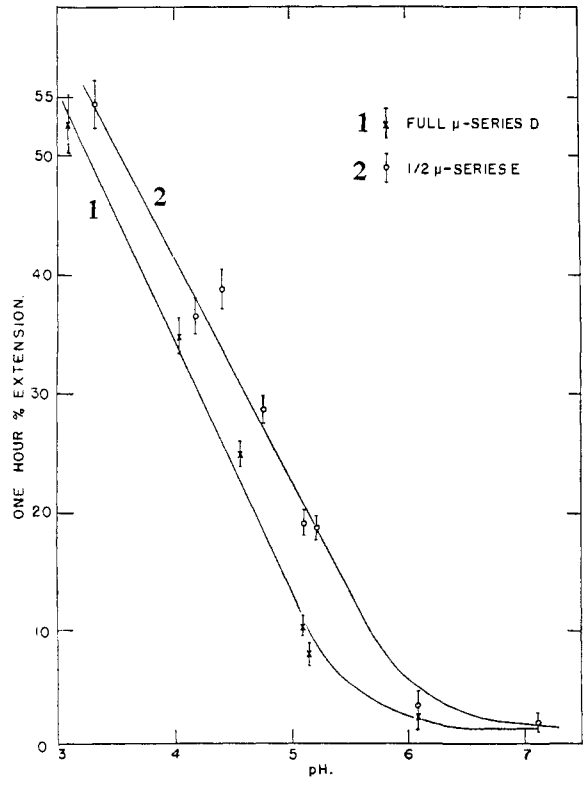


Fig. 3.—Effect of ionic strength of buffer on the rate of penetration of human γ-globulin into mixed films of cardiolipin–cholesterol (1:1 molecular basis) at 18.5 dynes/cm. (experimental conditions in Table I).

(40) H. T. S. Britton, "Hydrogen Ions," 3rd Ed., London, 1942, p. 304.

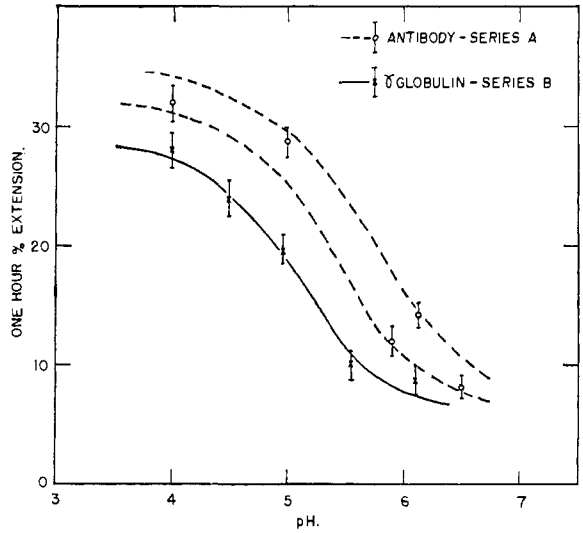


Fig. 4.—Penetration rate of Wassermann antibody and human γ-globulin into cardiolipin monolayers at 18.5 dynes/cm. (experimental conditions in Table I).

B. The Experimental Results.—The comparison of specific and non-specific surface reactions was made by studying the penetration of human γ-globulin and Wassermann antibody into monolayers of cardiolipin, 1:1 cardiolipin-lecithin and 1:1 cardiolipin–cholesterol (molecular ratios). For the system protein–cardiolipin (Fig. 4) it is seen that the dependence of the rate of penetration into the monolayer upon *p*H is the same for human γ-globulin and Wassermann antibody, although the antibody appears to react slightly more rapidly at *all* *p*H's. However, for the reaction of protein with monolayers of 1:1 cardiolipin–cholesterol (Fig. 5) and 1:1 cardiolipin–lecithin (Fig. 6), the picture is quite different. In both these cases the

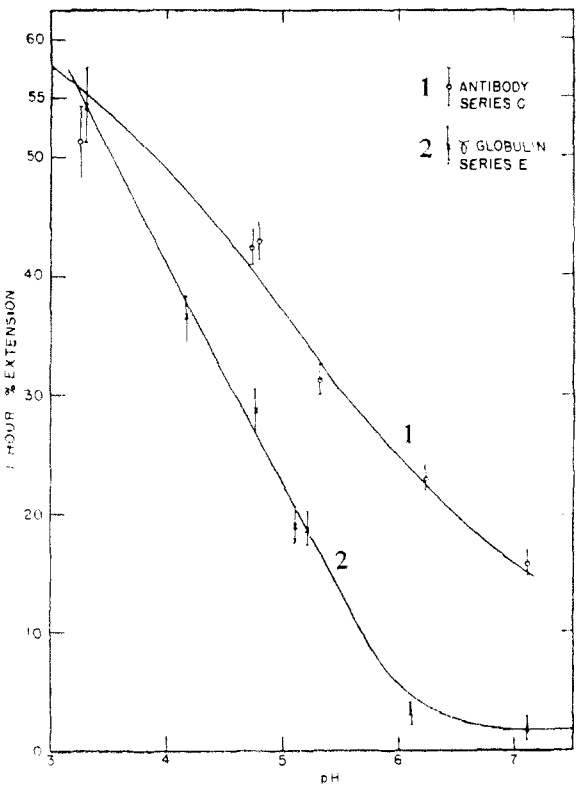


Fig. 5.—Penetration rate of Wassermann antibody and human γ-globulin into 1:1 cardiolipin–cholesterol monolayers at 18.5 dynes/cm. (experimental conditions in Table I).

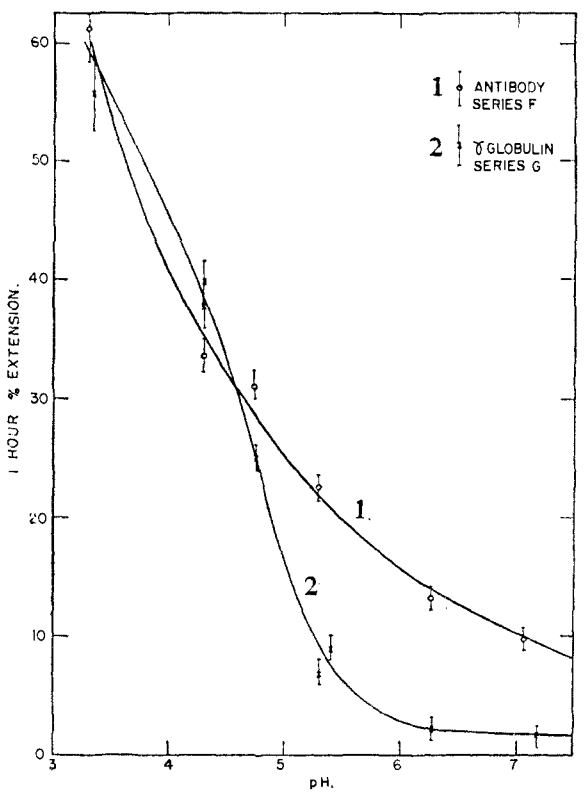


Fig. 6.—Penetration rate of Wassermann antibody and human γ-globulin into 1:1 cardiolipin–lecithin monolayers at 18.5 dynes/cm. (experimental conditions in Table I).

immune and normal γ-globulin differ in the dependence of their rates of penetration, the divergence becoming most marked in the *p*H region of 5.5 to 7. The experimental conditions are summarized in Table I. The experiments using antibody were not performed in any order of increasing or decreasing *p*H, and the antibody titer (Kahn) was measured throughout their duration. The experiments of series A were made with a different antibody preparation from those of series C and E, which were performed on the same material. Both preparations retained their activity, as judged by their Kahn titre, throughout the experiments.[40]

Discussion

In view of the fact that the reactants in the monolayer and substrate are charged molecules, the electrical interactions are bound to play a large role in determining the variation of the rate of penetration with *p*H. One problem of following the specific hapten–antibody reaction by the monolayer technique is, therefore, whether the charge-mediated interaction forms too intense a background against which the specific reaction cannot be distinguished. On the other hand, it appears unlikely that this is the case, since in the flocculation tests the haptenic particles are also charged and yet their reaction with antibody proceeds in the presence of an excess of non-specific protein. In general one may expect that at *p*H values distinctly acid and alkaline to the isoelectric point, the attractions and repulsions, respectively, of charged monolayers and protein will outweigh all other factors. Figures 5 and 6 show that this tendency is indeed obeyed. However, in the isoelectric region the different mechanisms of the specific and charge-mediated interactions should result in considerably different rates of penetration. It is not sufficient, though, that the penetration of antibody and normal γ-globulin into the monolayer should be compared at a single *p*H value near the isoelectric region, say at *p*H 6. Several circumstances prevent one from using the comparison of the numerical values of the rates of penetration of antibody and γ-globulin into the monolayers as a criterion for the existence of a specific reaction. First, the extinction coefficient of the immune and normal γ-globulin have been *assumed* to be equal. Secondly, the procedure for preparing the antibody[31] makes it inevitable that the material should be contaminated with surface-active impurities. If it were possible to remove the antibody activity without altering protein structure, then the penetration rate of antibody could be compared before and after denaturation. However, this cannot be done. Finally, γ-globulin is electrophoretically inhomogeneous. At any *p*H the distribution of charged species in the antibody and γ-globulin will, in all probability, not be the same. The present procedure of comparing the penetration rate of immune and normal γ-globulin into the lipid monolayer is therefore necessary. The criterion for the existence of a specific reaction is the appearance of differences in the *p*H dependence of antibody and γ-globulin penetration into the monolayer.

In terms of this criterion, the following interpretation is given to the experimental data. Figure

4 shows that there is only a slight difference in the rates of penetration of antibody and γ-globulin into pure cardiolipin at the same *p*H and that the dependence of rate upon *p*H is nearly the same. However, Figs. 5 and 6 show that both mixed films of 1:1 cardiolipin–cholesterol and 1:1 cardiolipin–lecithin distinguish between antibody and γ-globulin. It is therefore concluded that 1:1 mixtures of cardiolipin with lecithin, and cardiolipin with cholesterol are haptens to Wassermann antibody, while pure cardiolipin is not. At the same time, cholesterol or lecithin alone are *not* haptenic either.[41] In other words, either cholesterol or lecithin may complete the haptenic group of cardiolipin when present in mixed monolayers in equimolar proportions. This effect can be the result of two circumstances:

(1)—The haptenic group is polar, and the cholesterol and lecithin polar groups both may complete a configuration which is specific for Wassermann antibody. The surface pressure of the penetration experiments is sufficiently high, that were the specific reaction to involve the hydrocarbon chain, it would never be detected by this method. However, the cholesterol and lecithin polar groups are completely different. Should they both complete the specific grouping of the hapten it would have to be considered something of a coincidence.

It is of interest to mention, at this point, certain bulk experiments, that is, experiments with "antigen" suspensions, which were performed when it was found that there is no specific interaction between the antibody and cardiolipin in the surface experiments. The purpose of these studies was to find out qualitatively but relatively rapidly what effects certain adducts had upon the ability of cardiolipin to remove antibody activity from syphilitic sera. Accordingly "antigen" suspensions were made containing cardiolipin and one of a number of other substances such as cholesterol, lecithin, various solid and liquid long-chain aliphatic alcohols, bromides, amines and acids, in proportions that were modeled after a recipe of Kline[27] for antigens made from cardiolipin, cholesterol and lecithin. Their ability to reduce or remove antibody titer of syphilitic sera was compared.[42] Lack of space prevents a detailed description of these experiments, but the results show that while cardiolipin alone does not remove or inhibit the Wassermann antibody of syphilitic sera, it can do so when mixed with cholesterol, lecithin or solid long-chain alcohols. It is inactive in the presence of aliphatic acids, bromides, amines and most of the liquid long chain alcohols investigated. Conclusions drawn from this type of study should be tested in monolayer studies; since the bulk experiments suggested that cholesterol and lecithin might confer specificity toward Wassermann antibody upon a cardiolipin monolayer, the penetration experiments with mixed films were made. The inference drawn from the bulk experiments was verified. We might allow this to encourage us to predict also that further work will show that other polar molecules such as long-chain aliphatic alcohols, when mixed with cardiolipin, may also complete the haptenic requirements of Wassermann antibody, and that the physical state of the monolayer may influence its ability to undergo specific reaction with its homologous antibody.

(2)—The effect of cholesterol and lecithin is merely one of spacing cardiolipin molecules suitably. In support of this, is the fact that at 18.5 dynes/cm. the molecular areas of cholesterol and lecithin are 41 and 46 Å.^{2}/molecule, respectively,[35] while that of cardiolipin is 230 Å.^{2}. However, the following experiment leads one to believe that there is little or no compound formation between cardiolipin and cholesterol: The force–area curve of a mixed film of 1:1 cholesterol–cardiolipin on *p*H 5 buffer shows lower compressibility than the corresponding curve for pure cardiolipin as is to be expected from the addition of cholesterol, which gives a condensed monolayer with a limiting area of 40 Å.^{2} per molecule. The area for one molecule of each component in the mixed film is only about 6% less than the sum of the individual molecular areas in pure monolayers. With such a compressible substance as cardiolipin, the cholesterol should show a zero or even negative partial specific area in case of firm interaction. In the absence of compound formation, it is difficult to see how any effective regular spacing can be achieved.

Further studies, however, will demonstrate whether higher proportions of cholesterol or lecithin make the haptenic layer even more strongly antigenic. The qualitative experiments on antigen suspensions have also opened another line of investigation, namely, the elucidation of the haptenic group by substitution of different compounds in the monolayer.

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(41) M. Pangborn, *Disc. Far. Soc.*, No. 6, 110 (1949).

(42) A somewhat related study of non-specific precipitation was undertaken by Mackie and Anderson[43] and Anderson.[44]

(43) T. J. Mackie and C. G. Anderson, *J. Path. Bact.*, **44**, 603 (1939).

(44) C. G. Anderson, *Biochem. J.*, **32**, 282 (1939).