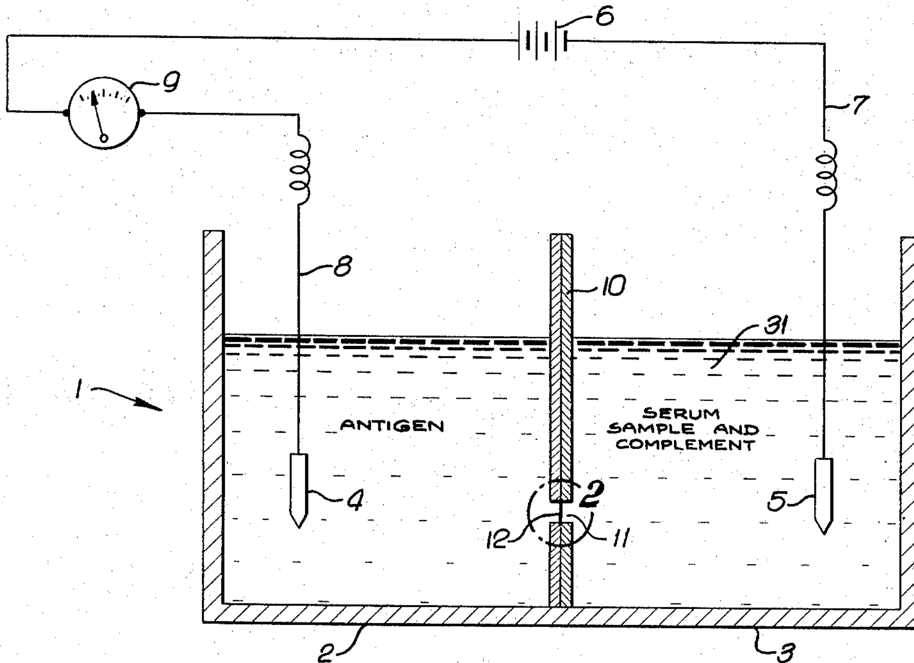
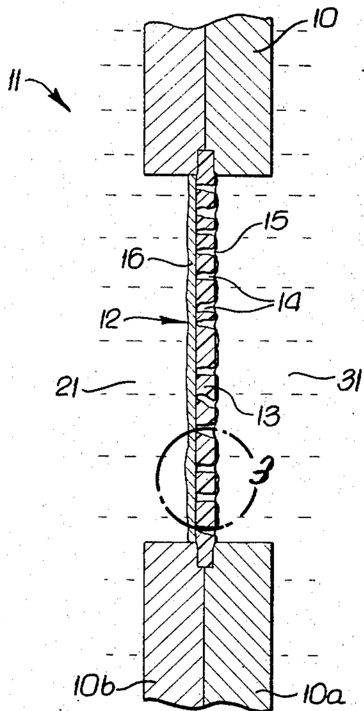
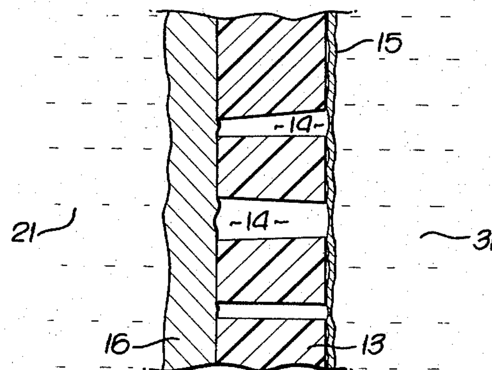
March 26, 1974 J. D. ALEXANDER ET AL 3,799,743

STABLE LYSIS RESPONSIVE LIPID BILAYER

Filed Nov. 22, 1971







United States Patent Office

3,799,743
Patented Mar. 26, 1974

3,799,743

STABLE LYSIS RESPONSIVE LIPID BILAYER

James D. Alexander, 1238 Barry Ave., West Los Angeles, Calif. 90025, and Per Barfort, 765 Oregon Ave., Palo Alto, Calif. 94303

Filed Nov. 22, 1971, Ser. No. 200,835
Int. Cl. B01k *3/10;* G01n *33/16;* G01r *27/02*
U.S. Cl. 23—253 R 24 Claims

ABSTRACT OF THE DISCLOSURE

Porous support for lysis responsive lipid bilayer enables reliable detection of immune reactants in serum samples by contact of the sample with the bilayer and sensing of lysis response.

BACKGROUND OF THE INVENTION

Field of the invention

This invention has to do with simple and sensitive detection of immune reactant in a sample. More particularly, the invention is concerned with improvements in the stability, reliability and sensitivity of lysis responsive lipid bilayers used in detection of immune reactant component e.g. in sera. In its essential aspects, the invention provides a highly stable yet sensitive lipid bilayer arrangement useful in the detection of immune principle in samples.

Prior art

It has been suggested that the electrical resistance of a lipid bilayer in a conductive solution can be lowered by effecting an immune reaction on the bilayer surface, e.g. by coupling antibody and antigen in the presence of complement; the reaction serving to locally lyse the bilayer at or near the reaction site, enabling transbilayer movement of ions and thus passage of current there, between solution immersed electrodes of different potentials. This is reported in Science 160, pages 1119–1121, (1968), by Per Barfort, one of the inventors herein.

The slowness to adoption of the lipid bilayer technique to immune analysis cannot be attributed to an absence of need for improvement over techniques now available. Present techniques are chemical and cumbersome or electronic and prohibitively expensive.

The fragility of lipid bilayers deriving from their noncrystalline nature and microscopic dimensions, e.g. less than 100 Angstroms thick, has, however, heretofore precluded practical instrumentation of the system for medical use.

SUMMARY OF THE INVENTION

It is a major objective of this invention to remedy the outstanding deficiency of lipid bilayer system for immune analysis by overcoming the bilayer fragility problem.

From this achievement flow manifold benefits including minimization of bilayer tearing and resultant spurious readings, lengthening of bilayer storage life, increased facility in forming bilayers and more reliable results through greater control of bilayer perforation.

In the mentioned Science article there is described apparatus for analysing a serum sample for immune i.e. antibody reactant comprising interfitting cups, each containing saline solution. The inner cup is provided at a sidewall with a circular opening of one square millimeter

area, located to be between the two solutions. A lipid bilayer is formed across this opening and electrodes at different potentials are inserted in the two solutions. Antigen is placed in the outer solution and the potentially antibody containing serum sample, together with complement, in the inner solution. If the suspected antibody for the antigen, and specifically that antibody, is present, a specific antibody-antigen couple is formed by the perfusion of antigen through the lipid bilayer and coupling at the inner solution side of the bilayer of the antigen and antibody. In the presence of complement, a complex of sequentially activated proteins, coupling of antibody and antigen produces a series of reactions at the site eventuating in a perforation of the bilayer; the reaction sequence being generically termed "the immune reaction," the details of which are beyond the scope of the present disclosure, but which for practical purposes may be considered to be a reaction akin to or identical with the selective cell destroying mechanism of the human body to ward off illness.

In general, the present invention improves upon and makes medically practical the discovery noted in the Science article, by supporting the lipid bilayer against tearing, through mechanical shock, while permitting the necessary perfusion of protein and perforation of the bilayer indicative of immune reaction.

Accordingly, the invention provides apparatus for the detection of an immune reactant, hapten antibody or antigen, potentially present in a sample. The apparatus comprises a lipid bilayer having, through containing antigen or antibody respectively, lysis response to immune reaction generated by the presence of its coreactant; means to contact the bilayer with the sample and complement, and signal means detectably indicating bilayer lysis response. The present invention provides the improvement comprising a nonlysable, coextensive porous support for the bilayer to which the bilayer adheres, the support having no pores greater than 10 microns in cross-section.

The pore sizes may be in the range of 10 microns to 0.1 micron and even as small as 1 angstrom to 1000 angstroms. The lipid bilayer, comprised of natural and artificial membranous lipid materials such as those hereinafter disclosed, may range between 40 and 100 angstroms in thickness across the support pores and be of greater or lesser thickness elsewhere on the support surface, and may or may not initially contain therein selected coreactant, e.g. antigen to be set against the sample. The support may be coated with agar or like gelatinous material on the surface opposite the lipid bilayer.

The porous support may comprise cellulosic, synthetic organic plastic, metallic or mineral material and particularly a plastic foil of e.g. polyolefin, poly vinyl halide, polyester or polystyrene and preferably polycarbonate film which is randomly porous with pores circular in cross-section and between 10 and 0.1 micron in diameter.

The invention contemplates a method of forming stable planar lipid bilayers which includes coating a solution of lipid in a volatile organic solvent onto a porous film having no pores greater than 10 microns, and drying the lipid across the pores by diffusion away from the lipid solution of the solvents for at least 20 minutes at temperatures between 10 and 65° C. The lipid may comprise a phospholipid and plasticizer therefor and be dissolved in

a solvent selected from chloroform, methanol and water mixtures thereof to a concentration between 0.1 and 10% weight by volume and thereafter dried to remove the solvent and form an integral layer.

In particularly preferred embodiments, the lysis response of the lipid bilayer is detected by electrical conduction through the bilayer. For this purpose the invention provides in apparatus for the detection of immune reactant in a sample comprising an immune-reaction lysable lipid bilayer extending in electrically insulative relation between first and second electrically conductive aqueous solutions, one of which contains complement and the sample to be tested for the presence of a first immune reactant, and the other of which contains a second immune reactant reactive with the first immune reactant, means applying a different potential to the first and second solutions, and means signaling current flow between the solutions through the bilayer; the improvement comprising an ion porous non-lysable coextensive support for the bilayer to which the bilayer adheres, the support having no pores greater than 10 microns in cross-section.

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawing:

FIG. 1 is a schematic view of apparatus according to the invention;

FIG. 2 is a detail view greatly enlarged of the lysable membrane portion, shown in FIG. 1; and

FIG. 3 is a further greatly enlarged detail view of the support and lipid bilayer system according to the invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Introduction

It has been known for many years that the combination of IgG or IgM antibody with a homologous membrane-bound antigen or hapten, in the presence of complement, can cause lysis of the membrane at or near the attachment site of the antibody molecule. This finding is the basis for the assay known as immune hemolysis.

Generally, it can be stated that any phospholipid-containing membrane which is physically stable at physiological values of pH, temperature, and ionic strength can be used as a medium for complement-induced immune lysis. Examples of biological membranes which have successfully been lysed by complement include human and sheep erythrocytes, mouse ascites tumor cells, rat peritoneal mast cells, Gram-negative bacterial cell walls from *Escherichia coli, Veillonella alcalescens, Shigella shigae,* and *Bacillus licheniformis.* Virus envelopes from infectious bronchitis virus have been subject to immune lysis, and lipopolysaccharide particles from Gram-negative bacteria (*Veillonella alcalescens* and *Escherichia coli*) exhibit the characteristic lesions after the immune reaction has occurred on their surface.

Artificial membranes which contain a phospholipid can be lysed by the same components which will lyse the biological membranes listed above. The incorporation of plasticizers in the lipid mixture tends to increase the time interval in which the membranes possess structural integrity without affecting the sensitivity of the immune response. It has been clearly demonstrated, for example, that the presence of cholesterol in biological or artificial lipid membranes (bilayers) does not alter the ability of complement to form holes in the membranes, and the appearance of the lysed membranes on electron micrographs is not affected.

The choice of phospholipid is limited to those sufficiently stable to last for the duration of complement-induced lysis without a comparable amount of unrelated (i.e. non-specific) lysis. Useful lipids have been found to be sphingomyelin or lecithin with cholesterol added. Planar bilayers may be formed by a wide variety of lipid mixtures which give membranes that are stable enough to last for the length of time required. Generally, a stable lipid membrane (i.e. a bilayer) is formed with the addition of a plasticizer to the lipid moiety, and the term lipid bilayer herein is intended to include mixtures of lipids per se and plasticizer.

In immune hemolysis assay noted above, the number of holes formed in a particular population of erthrocytes is measured by the amount of hemoglobin released from inside the cells. A recent modification uses the radioactive isotope Cr^{51} instead of hemoglobin as the indicator. The appearance of the characteristic holes on electron micrographs seems to indicate that an antigen-antibody reaction has activated complement at the membrane surface, but it is questionable whether it can be assumed that the holes (as seen by electron microscopy) actually represent functional lytic sites.

The electrical conductance of planar lipid membranes in the direction perpendicular to the membrane surface can be used as a measure of the structural integrity of the membrane. An intact membrane, consisting of phospholipid and a plasticizer, has a very low electrical conductance in a buffered NaCl solution between 30° and 40° C. The addition of any two of the following three moieties (a) antibody of type IgG or IgM, (b) homologous antigen or a hapten, and (c) complete complement, will not cause any permanent increase in the electrical conductance of the membrane. All three moieties can, however, induce immune lysis of the lipid bilayer if antibody and complement are present together in the aqueous phase at one side of the membrane. The antigen or hapten may either be present in the aqueous phase at the opposite side of the membrane or be a part of the membrane, or both. The electrical potential across the membrane (or, conversely, the electrical field) obviously must be limited so as to not cause a dielectric breakdown of the lipid membrane, with or without associated protein molecules. A potential not exceeding 20 mv. for a membrane between 40 A. and 70 A. thick will not significantly reduce the lifetime of a lipid membrane. The current passing through the membrane is proportional to the electrical conductance when the membrane potential is held constant.

The functional lysis of a lipid bilayer membrane is done by complement. The lysis may be induced by a homologous antigen-antibody reaction at the membrane or by an analogous non-homologous reaction, and subsequent activation of the first component of complement. If the complement is complete, the necessary divalent cations are present, and the temperature, pH and ionic strength of the aqueous solution are close to physiological values, the complement will eventually lyse the membrane. A similar lysis can be initiated by intermediary compounds in the complement reaction, thus eliminating the need for an antigen-antibody reaction.

A planar lipid bilayer membrane separating two aqueous phases constitutes a favorable system for the detection of immune lysis. The intrinsic conductance of the membrane is very low as mentioned, and the sensitivity to lytic activity can be very great. The antigen or hapten moiety can be a part of the lipid mixture used to form the membrane if this compound is hydrophobic, or it may be suspended or dissolved in one of the aqueous phases if it is a hydrophilic substance. This freedom does not exist with detection methods which require incorporation of the antigen or hapten into the membrane structure.

The use of immune lysis as an assay for an antigen-antibody reaction necessitates a certain structural stability of the membrane material. It can be stated that the potential sensitivity of the method is inversely proportional to the nonrelated lysis (i.e. background lysis) of the membrane system. The stability of an unsupported lipid film with an area of the order of 1 $mm.^2$ is not sufficient to

assure a membrane lifetime long enough to complete an assay, let alone storage before usage.

Accordingly, and as noted above, the present invention provides a porous support for the lipid bilayer. In the course of developing the support technology it has been observed that suitable support materials will be adherent to the lipid bilayer or adhered to by the bilayer and will have no pores greater than 10 microns in cross-section. The geometry of the pores is not narrowly critical with circular, rectangular, including square, and other polygonal (in cross section) pores being suitable. Circular cross-section pores are particularly preferred. In addition, the pore interior walls should be regular and not filled with e.g. fiber ends lying in random planes transverse to the pores longitudinal axis. The length or depth of the pores is not critical and will ordinarily be greater than the expected thickness of the lipid bilayer which is to extend across the pore opening. For suitable strength, a thickness of 0.25 to 5 mils in a plastic film or foil is highly satisfactory with 0.5 to 1.5 mil films of polycarbonate resin being very advantageous.

It is the function of the porous support to carry the lipid bilayer in the liquid test environment and accordingly the film should be insoluble in and resistant to degradation by the ambient liquid systems.

Suitable porous support materials which may be mentioned among others are synthetic organic plastic materials, either normally solid thermoplastic resins or thermosetting resins, which form electrically nonconductive films supported or self-supporting, or webs of fibers of such materials, either woven or nonwoven which permit pore forming aperturing meeting the above criteria of size, less than 10 microns, and freedom from abrupt dimensional variations along the pore length. Specific synthetic organic plastic materials among thermoplastic polymers and resins are polyolefins such as polyethylene, polypropylene, ethylene-propylene copolymers, polystyrenes, ethylene-styrene copolymers, ethylene-butene copolymers, polybutenes, ethylene-hexene copolymers, styrene-divinyl benzene copolymers, styrene-acrylonitrile copolymers, styrene butadiene copolymers, styrene-acrylonitrile-butadiene copolymers, polyvinyl chloride, ethylene-vinyl chloride copolymers, polyvinyl acetates, acrylic polymers such as poly (methyl methacrylate), poly (ethyl acrylate) ethylene-ethyl acrylate copolymers, polyvinyl butyrates and the like wherein one polymer forming moiety contains the olefinic unsaturation i.e. >C=C< and constitutes 25% or more by weight of the plastic material.

A further grouping of thermoplastic, synthetic organic materials are polyesters, particularly polycarbonate resins, products of the reaction of difunctional phenols, particularly 2,2-bis (4-hydroxyphenyl) propane and a carbonate radical precursor such as phosgene or diallyl carbonate. This just mentioned material is available commercially in a microporous film version having a pore size less than 10 microns and this is the preferred material for the bilayer support herein.

Other suitable specific polymeric materials, thermoplastic or thermosetting which may be mentioned are: acetals, alkyds, allyls, amino resins, chlorinated polyethers, polyhydroxyethers, polyphenylene oxides, parylenes, nylons (polyamides), polyimides, phenolics, e.g. phenol-aldehyde resins, polysulfones, polyisocyanates (polyurethanes), polyesters and silicones, in molecular weights to be film forming (supported or self-supporting) and stably porous, when pores have been formed therein, and adherent to lipid bilayer, either inherently or after suitable treatment.

Other porous support materials include cellulosic materials e.g. paper and cellophane (regenerated cellulose) suitably treated for resistance to the liquid environment; metals, where support conductivity is not a factor or where the support may be insulated against conductance. Suitable metals will include, tinfoil, aluminum, copper, lead, zinc, gold, silver, brass and the like; as well as minerals such as glass.

Techniques for forming the support films or foils are conventional. Pore formation may be electrical, mechanical, by nuclear radiation (e.g. cobalt 60 radiation particularly with the mentioned polycarbonate film) or otherwise to produce holes in the film or foil support 10 microns or less in cross-section.

Supported on the porous support and hered thereto is the lipid bilayer. Lipid bilayer formation is known and is carried out herein substantially in the manner of the science article noted above, save that the lipid solution is applied over the porous film microopenings and not the relative macroopening of 1 millimeter. The resulting laminate is unique in comprising a lipid bilayer alternately supported and unsupported randomly across its extent in two directions and a porous film-like support defining the areas of relative support and non-support.

Specific lipid bilayers preferred herein comprise sphingomylen, lecithin and/or phosphatidal ethanolamine and as plasticizers therefor α-tocopherol, cholesterol, n-decane and/or n-tetradecane. Also usable is any lipid mixture forming a bilayer. Lipids in this context has reference to hydrophobic/hydrophilic molecules such as fatty acids and soaps, neutral fats, waxes, steroids and phosphatides, with "fat" having reference to esters of glycerol with fatty acids e.g. acids both saturated and unsaturated having from 12 to 24 carbon atoms. Such materials are organic, insoluble in water, soluble in alcohol and greasy feeling.

The natural and synthetic biological membranous materials noted above may be employed as lipid bilayers together with or in substitution for materials just enumerated.

In the drawing, an apparatus according to the invention is depicted. In FIG. 1 vessel **1** is shown to comprise left tank **2** and right tank **3**. A left electrode **4** is immersed in tank **2** and right electrode **5** is immersed in tank **3**. Electrode **5** is connected to battery **6** by line **7**. Electrode **4** is connected to the opposite side of battery **6** by line **8** which includes an ammeter **9**. Tanks **2** and **3** are separated by a wall **10** which is apertured at **11** to contain a support lipid bilayer laminate **12**. Aqueous, saline solutions **21**, **31** are contained in tank **2** and tank **3**, separated by wall **10** and by laminate **12** in aperture **11**. Electrodes **4** and **5** are maintained at different potentials to create a potential gradient across laminate **12**. The laminate **12** is normally nonconductive and remains so while the lipid bilayer remains intact.

In FIG. 2, the aperture **11** is shown in greater detail. The laminate **12** is seen to comprise a plastic film support **13** having multiple relatively small, randomly located pores **14**, the lipid bilayer **15** and across the support therefrom an agar gel layer **16**. The wall **10** is seen to comprise right and left sections **10*a*** and **10*b*** joined by means not shown, which may each be Mylar/polyethylene laminates heat sealed together to clamp the film **13** therebetween to support the film in the aperture **11**. The adherence of the bilayer to the film is shown in FIG. 2. The stretching of the lipid bilayer over the pores **14** is best shown in FIG. 3 where the bilayer is attenuated to a stable free (unsupported) state e.g. at a thickness of less than 100 angstroms and typically between 40 and 70 angstroms.

EXAMPLE

Apparatus was assembled with interfitting cups forming the separate tanks rather than side-by-side tanks illustrated in the drawing. A 2 millimeter disk film of polycarbonate 0.4 mil thick having plural 0.4 micron randomly distributed pores was suspended tautly in an aperture in the inner cup with a plastic film formed of Mylar and polyethylene.

Saline solution was prepared comprising 0.1 M NaCl buffered to pH 7 with $NaPO_4$ and held at 37°.

A lipid bilayer forming mixture was prepared at 25° C. from D,L-α-tocopherol, methanol and chloroform in a weight ratio of 5, 2, 3. To this mixture, sphingomyelin at 2.5% (weight to volume) was added.

The saline solution was divided and placed in the inner and outer cups. The bilayer forming solution was brushed onto the disk in immersed condition. The bilayer formed by diffusion of the solvent, chloroform and methanol, into the aqueous phase, leaving the α-tocopherol and sphingomyelin as a bimolecular layer on the disk and across the disk pores at a thickness of about 70 angstroms.

Electrodes connected to a 20 millivolt power supply were immersed in the inner and outer saline solutions and current passage therebetween monitored with an ammeter connected in series with the power supply. Coating of the disk was continued until no current flow was detected indicating an insulative barrier had been formed by the bilayer across the disk pores which of course are of a size to pass ions and immune reactants such as antigen, hapten, antibody and complement.

The apparatus was tested by mixing the antigen insulin with agar and applying this mixture onto the outer side of the disk. A sample of guinea pig serum known to contain antibody to insulin, and complement (ex. guinea pig serum) were added to the inner saline solution. The ammeter was monitored for current flow and after two minutes an increasing current was detected, signalling perforation of the bilayer.

While fully developed in the literature, in the interests of completeness of disclosure, it may be added here that the movement of antigen from the outer solution or the agar gel or even from the bilayer forming mixture through the bilayer to locally site on the antibody side of the bilayer enables coupling of the antibody to the antigen, which coupling, activates complement, the complex of sequentially activatable proteins described in the literature, which functions when activated to lyse, i.e. perforate the bilayer and thus permit ion movement therethrough. The location of the lytic site is not nesessarily at the pore initially, but hole growth eventually opens a pore to ion movement.

As is known antibody-antigen reactions are highly specific. Thus the present invention enables a rapid inexpensive, specific diagnostic tool for immune reactant detection. The physician need only select the antigen for the antibody sought to be detected (or vice versa) and contact the two across the lipid bilayer, in the presence of complement to achieve the lysis which can be detected colorimetrically, electrically or otherwise.

The discovery of means to support the bilayer enable pre-preparation of e.g. various antigen specimens which may be drawn on as needed, as well as eliminating spurious results occasioned by mechanically induced defects in the bilayer, e.g., by jarring the apparatus.

We claim:

1. In apparatus for the detection of an immune reactant potentially present in a sample, which comprises a lipid bilayer having lysis response to immune reaction generated by the presence of said reactant, means to contact the bilayer with said sample and complement, and signal means detectably indicating bilayer lysis response, the improvement comprising a nonlysable, coextensive porous film support having no pores greater than 10 microns in cross-section, to which the bilayer is adhered in alternately supported and unsupported relation randomly across its extent in two directions.

2. Apparatus according to claim **1** in which the support pores range in size from 10 microns to 0.1 micron.

3. Apparatus according to claim **1** in which the support pores range in size from 1 angstrom to 1000 angstroms.

4. Apparatus according to claim **1** in which said lipid bilayer ranges in thickness between 40 and 100 angstroms across support pores.

5. Apparatus according to claim **1** in which the lipid bilayer comprises phospholipid and plasticizer therefor.

6. Apparatus according to claim **1** in which said lipid bilayer contains immune reactant reactive with immune reactant to be detected in said sample.

7. Apparatus according to claim **1** in which said porous support comprises cellulosic, synthetic organic plastic, metallic, or mineral material.

8. Apparatus according to claim **7** in which said porous support comprises cellulosic material.

9. Apparatus according to claim **7** in which said porous film support comprises metallic foil.

10. Apparatus according to claim **7** in which said porous film support comprises mineral material.

11. Apparatus according to claim **7** in which said porous film support comprises a flexible synthetic organic plastic foil.

12. Apparatus according to claim **11** in which said foil comprises a self-supporting film forming polymer selected from the group consisting of polyolefins, poly vinyl halides, polyesters and polystyrene.

13. In apparatus for the detection of an immune reactant potentially present in a sample, which comprises a lipid bilayer having lysis response to immune reaction generated by the presence of said reactant, means to contact the bilayer with said sample and complement, and signal means detectably indicating bilayer lysis response, the improvement comprisng a nonlysable, coextensive porous support for the bilayer to which the bilayer adheres, said support having no pores greater than 10 microns in cross-section, and said support being coated with gelatinous medium opposite the lipid bilayer.

14. Apparatus according to claim **13** in which said support comprises polycarbonate resin.

15. In apparatus for the detection of an immune reactant potentially present in a sample, which comprises a lipid bilayer having lysis response to immune reaction generated by the presence of said reactant, means to contact the bilayer with said sample and complement, and signal means detectably indicating bilayer lysis response, the improvement comprising a nonlysable, coextensive porous polycarbonate resin film support for the bilayer to which the bilayer adheres, said support having no pores greater than 10 microns in cross-section.

16. Apparatus according to claim **15** in which said polycarbonate film is randomly porous and said pores are circular in cross-section and between 10 and 0.1 micron in diameter.

17. Apparatus according to claim **15** in which said support is coated with gelatinous medium opposite the lipid bilayer.

18. In apparatus for the detection of immune reactant in a sample which comprises an immune-reaction-lysable lipid bilayer extending in electrically insulative relation between first and second electrically conductive aqueous solutions one of which contains complement and sample to be tested for the presence of a first immune reactant and the other of which contains a second immune reactant reactive with the first immune reactant, means applying a different potential to the first and second solutions, and means signaling current flow between said solutions through said bilayer, the improvement comprising an ion-porous nonlysable coextensive support for the bilayer to which the bilayer adheres, said support having no pores greater than 10 microns in cross-section.

19. Apparatus according to claim **18** in which the support pores range in size from 10 micron to 0.1 micron.

20. Apparatus according to claim **18** in which the support pores range in size from 1 angstrom to 1000 angstroms.

21. Apparatus according to claim **18** in which said lipid bilayer comprises a coating on said support and ranges in thickness between 40 and 100 angstroms across support pores.

22. Apparatus according to claim **18** in which said lipid bilayer contains immune reactant reactive with immune reactant to be detected in said sample.

23. Apparatus according to claim **18** in which the lipid bilayer comprises a phospholipid selected from sphingomyelin and mixtures thereof and a plasticizer therefor selected from α-tocopherol, n-decane, n-tetradecane and cholesterol and mixtures thereof.

24. Apparatus according to claim **18** in which said porous support comprises cellulosic, synthetic organic plastic, metallic, or mineral material.

References Cited

UNITED STATES PATENTS

2,494,559	1/1950	Jubanowsky	117—167 X
2,916,392	12/1959	Chapin et al.	117—167 X
3,393,081	7/1968	Bogle	117—167 X
3,607,700	9/1971	Tosteson	204—195 L

OTHER REFERENCES

Barfort et al., Science, 160, 1119–1121 (1968).

ROBERT M. REESE, Primary Examiner

U.S. Cl. X.R.

117—143, 167; 195—103.5; 204—296; 324—62