123. Immunological Reactions Carried out at a Liquid-Solid Interface

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Résumé. Des γ-globulines humaines et des polysaccharides de pneumocoques de types III et VIII («antigènes») adsorbés en couches minces de 5 à 30 Å d'épaisseur sur des plaques métallisées par du chrome, sont capables d'adsorber spécifiquement des couches d'anticorps pouvant aller jusqu'à plusieurs centaines d'Å d'épaisseur. Les vitesses d'adsorption d'anticorps homologues par les couches de globulines ou de polysaccharides déposées sur les plaques furent mesurées avec un ellipsomètre enregistreur. Avec des plaques non recouvertes préalablement d'antigène, les couches adsorbées après immersion des plaques dans l'antisérum étaient extrêmement minces, d'environ 12 Å, ce qui montre bien que les adsorptions observées en présence d'antigène ont un caractère spécifique. De très grandes différences dans les vitesses d'adsorption sont observées selon que les plaques sont respectivement sèches ou mouillées au moment de l'immersion. Les plaques mouillées adsorbent les anticorps plus rapidement et en une couche plus épaisse que les plaques sèches pourvu que la densité d'antigène présent sur la plaque soit suffisamment élevée. Cette condition était réalisée par l'emploi de solutions d'antigène de 10^{-3} g/ml pour revêtir les plaques d'une couche d'antigène. Cette différence dans la vitesse d'adsorption peut s'expliquer si l'on formule l'hypothèse qu'il y a coopération entre les molécules d'antigène lorsqu'elles sont suffisamment voisines, ce qui permet une interaction à grande distance favorisant la diffusion des molécules d'anticorps à travers le film d'eau adhérant à la plaque, au détriment des globulines non spécifiques du sérum.

Lorsqu'un faible courant électrique (300 μA) est utilisé pour l'adsorption des antigènes et des anticorps, de très faibles concentrations d'antigène descendant jusqu'à 10^{-14} g/ml et parfois jusqu'à 10^{-15} g/ml peuvent être décelées.

Ces resultats ne sont obtenus qu'avec des plaques révélant par diffraction d'électrons la présence de cristallites de chrome partiellement orientés, d'au moins 50 Å de diamètre, ce qui confirme l'hypothèse que des phénomènes de coopération entrent en jeu dans ces réactions immunologiques à une interphase liquide-solide.

More than twenty years ago, we showed that monolayers of proteins spread on the water surface of a *Langmuir* trough and then transferred to metallized glass slides were capable of adsorbing specifically corresponding antibodies [1] [2]. In spite of the loss of the secondary and tertiary structure of the protein molecules, the layers about 8 Å thick, had maintained their specific interaction with antisera. Protein molecules could also be adsorbed directly on a slide from a dilute aqueous solution. In this case they were not surface denatured and the thickness of the average layer was 20 to 30 Å depending on the molecular weight of the protein. A layer of antibodies as thick as 80 Å could be specifically immobilized on the subjacent layer of adsorbed antigen. The antigenic layers were not limited to proteins. Similar experiments were carried out with adsorbed layers of polysaccharides from Types I, III or VIII *pneumococcus*. The underlying layer of polysaccharide was 4 to 5 Å thick only, but the layer specifically adsorbed from the antisera could be as thick as 600 Å [2]. In other experiments we studied the action of enzyme solutions on antigenic layers in the presence of a membrane of inactive material a few hundred Å thick deposited on top of the antigenic layers and separating them from the enzyme solution. The results clearly

indicated that the interaction could not be explained in terms of ordinary diffusion of the enzyme molecules through the membrane. A 'forced' diffusion mechanism had to be assumed, in other words, forces other than those resulting from a concentration gradient were at play to bring together enzymes or antibodies and protein layers [3]. To obtain a better understanding of the mechanism involved, the kinetics of adsorption of antibodies on slides coated with antigen were studied. The major part of this work will be published elsewhere, but some new and far-reaching facts, which have a deep significance for the fundamentals of these reactions, warrant their presentation in this paper.

Experimental. – Glass slides plated by evaporation with a layer of chromium 4000 Å thick were used as anchorage.

The rates of adsorption were measured with a recording ellipsometer [4] capable of measuring the thickness of an adsorbed layer during the course of its formation. The antigen coated slides, upon which the adsorption of antibodies occurred, were dipped into a cuvette containing a few tenths of a ml of the antiserum solution; the cuvette was placed in a tight fitting water jacketed brass cell, with narrow entrance and exit slits. The incident beam of plane polarized light which became elliptically polarized after reflection from the slide was analyzed with a $\lambda/4$ plate and an analyzer. Thus the reflection occurred at a water-solid interface instead of an air-solid interface as in the ordinary ellipsometer. At the beginning of an experiment the $\lambda/4$ plate and analyzer were so adjusted as to extinguish the reflected elliptically polarized light. Both $\lambda/4$ plate and analyzer remained thereafter stationary; as the adsorption proceeded the intensity of the reflected light which entered the photomultiplier tube increased gradually. The instrument, therefore, played the role of a photometer. Calibration curves were prepared with slides coated with films of known thickness, such as Ba stearate, plunged into the cuvette filled with appropriate aqueous solutions. The relationship between the thickness adsorbed and the intensity of the reflected light was nearly linear, if, at the beginning of an experiment, the ellipticity of the reflected light was not quite fully compensated. This permitted an easy correlation between increase in light intensity and corresponding thickness of the adsorbed layer. The signal from the photomultiplier tube was amplified and simultaneously recorded on a 10″ strip chart recorder (*Photovolt*) and a tape recorder. The curves presented in this article were obtained with the chart recorder. Data obtained with the tape recorder that allow the measurement of fast rates of reaction will be presented elsewhere. The rates of adsorption of the antigen were not studied. The antigen was adsorbed for a constant time of the order of a few minutes.

The slides were either cleaned with a watery paste of cerium oxide, and rinsed, or used without cleaning, as they came out of the evaporation chamber. When the concentration of the antigen solution was large ($> 10^{-6}$ g/ml), the results were the same whether the slides had been cleaned or not, but immunologic reactions could be detected with much more dilute solutions of antigen if the slides had not been cleaned first.

For all the experiments reported in this paper the antisera were diluted one to ten in veronal buffer pH 7.5 0.03 M. Two systems were mainly investigated, human γ-globulin-antihuman γ-globulin rabbit serum, and the polysaccharides from Type III and Type VIII *pneumococcus* and their respective rabbit antisera.

Human γ-globulin-Antihuman γ-globulin. – The human globulin was adsorbed for three minutes from a veronal solution containing 10^{-3} g/ml of globulin. The layer adsorbed was 30 Å thick. Representative measurements of rates of adsorption from the immune serum on such coated slides are shown in Fig. 1 in which the thickness is plotted against time. Curve *a* was obtained when, after the antigen adsorption and washing, the slide was introduced wet into the antiserum solution. Curve *b* was obtained when the slide was dried before immersion into the antiserum. Proper controls were made by measuring the thickness adsorbed from the same antiserum on a slide coated with an adsorbed layer of a heterologous antigen, in this

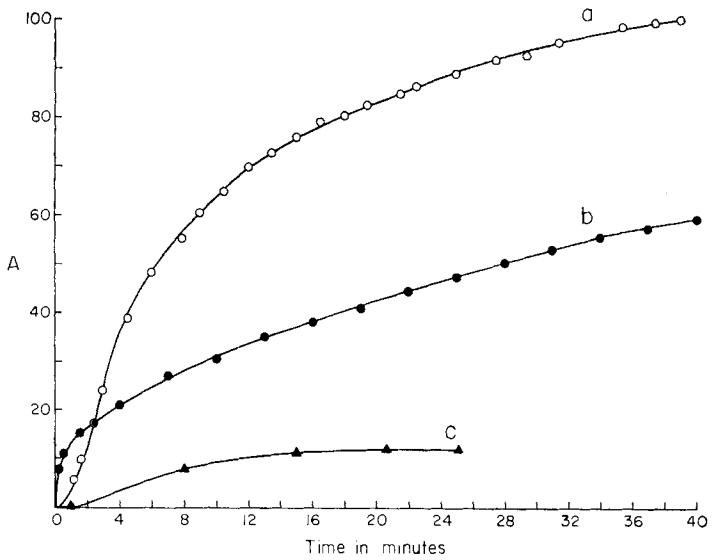

Fig. 1. *Adsorption from an antiserum diluted 1/10 in veronal against human γ-globulin on a slide coated with human γ-globulin*

Curve *a*, slide introduced wet, and curve *b*, slide introduced dry into the anti-serum

case rabbit globulin. The adsorption observed is non-specific in this case and is represented by curve *c* of Fig. 1. For this control experiment the slide coated with the rabbit globulin was introduced wet into the antiserum. It is evident that the non-specific adsorption ($\simeq$ 12 Å in 25 min) is a very small fraction of the adsorption represented by curves *a* and *b*.

As seen from the curves, the thickness adsorbed on a slide introduced wet into the antiserum was 83 Å after 15 minutes, whereas it was only 37 Å in the same time interval if the slides had been dry when immersed into the antiserum. This result was most unexpected and it has deep implications for the understanding of the mechanisms involved. A close examination of the two curves shows that at the very beginning of the adsorption, the rate is slower on a slide introduced wet into the antiserum. This is understandable since it takes a finite time for the molecules of antibodies to diffuse through the layer of water immobilized on a wet slide, whereas a dry slide in a fraction of one minute is already coated with a thickness close to 15 Å. However, once the barrier offered by the water has been overcome the rate of adsorption on a slide introduced wet overtakes that observed on a slide which was originally dry. This behavior appears to be quite general and so far proved independent of the nature of the antigen.

Polysaccharide from *pneumococcus* III and IV - Antipolysaccharide. – A slide coated with a 5 Å layer of polysaccharide from Type III or VIII *pneumococcus* could adsorb in one minute a layer of antibodies 130 Å thick if the slide had been kept wet after the adsorption of the polysaccharide, whereas only 77 Å of antibodies were adsorbed if the slide had been dried after the adsorption of the polysaccharide. In the case of the polysaccharide the thickness adsorbed from the antiserum was very much

dependent on the pH of the solution of the polysaccharide. The maximum thickness of the layer of antibodies was obtained when the pH of the antigen solution was around 2.2. A slide coated with polysaccharide from a solution of pH 2 and kept wet before immersion in the antiserum could adsorb a layer 204 Å thick in 5 minutes, whereas a layer only 110 Å thick was adsorbed in the same length of time on a slide dried before immersion.

The simple adsorption of human globulin under different conditions on metallized slides was investigated in order to obtain some understanding of the difference in behavior between a dry and a wet slide toward the specific adsorption of antibodies.

A layer of globulin 30 Å thick was rapidly adsorbed from a relatively concentrated solution (2×10^{-3}g/ml) on a slide introduced either wet or dry in the protein solution; the thickness did not increase with the length of immersion. If the slide after emersion and rinsing was kept wet and immersed again into the solution, no additional layer could be adsorbed. Many successive layers, however, could be adsorbed on top of each other if the slide was dried after each emersion. This behavior is characteristic of globulins, since multilayer adsorption could not be obtained with bovine serum albumin for instance. The results obtained with γ-globulin indicate that if a layer of water is present on the first adsorbed layer of globulin, this layer of water cannot be displaced to allow the adsorption of a second layer of globulin. The water acts as an insuperable barrier. When a 2% aqueous solution of methanol is used instead of water, the barrier becomes somewhat less efficient and a thickness of 9 Å could be adsorbed in one minute. When a layer of bovine albumin about 20 Å thick was first adsorbed on a slide, another globulin layer 20 Å thick could be adsorbed on top of it, if the slide was dry when introduced into an antiglobulin serum, but no adsorption whatever occurred if the slide had been introduced wet. This again shows that the globulin molecules of antiserum are unable to cross the barrier offered by the film of water adhering to the bovine albumin layer. However, when a globulin layer was substituted for the bovine albumin layer, then 25 Å of antibodies could be adsorbed from the antiglobulin serum in spite of the fact that the slide was introduced wet into the solution. The following conclusions can be drawn from these experiments: the interaction antibodies-antigen is strong enough to permit the antibody molecules to cross the barrier offered by the film of water and to react with the subjacent film of antigen, barrier which they could not overcome if the subjacent film was a heterologous protein. Thus, these results might offer a reasonable explanation of why antibodies are adsorbed much more rapidly on an antigen coated slide introduced wet rather than dry into the antiserum. The thin layer of water adjacent to the slide acts as a filter which prevents to a considerable extent the diffusion of the non-specific proteins, whereas the specific antibodies on account of the very strong interaction with the antigenic layer, most likely long range, cross the barrier of water to become specifically immobilized. The antigen coated surface of a dry slide can readily adsorb any globulin and since most of the adsorbable globulins of the antiserum are non specific, they will interfere with the specific adsorption of the antibodies, whose rate will be slowed down, thus reducing the total specific thickness adsorbed.

It is worth noting that if the interaction antigen-antibody is decreased, a slide thickly coated with antigen and immersed dry into the antiserum solution adsorbs a thicker layer than when immersed wet. This condition was realized in the following

example. There is a strong cross reaction between the systems polysaccharide Type III and polysaccharide Type VIII and their corresponding antisera. However, the affinity of the heterologous reaction is weaker than the homologous one, since the immunologic homologous reaction can be detected at much greater dilutions than the heterologous one. It was observed that a slide coated with polysaccharide VIII adsorbed a thicker layer from an antipolysaccharide III serum when immersed dry rather than wet. Similarly, a slide coated with polysaccharide III adsorbed a thicker layer from an antipolysaccharide VIII serum when immersed dry rather than wet. Under these conditions, the force acting on the antibodies to displace the layer of water was not sufficient to compensate for the initial more rapid adsorption which takes place on a dry slide.

It is important to notice that as the concentration of the antigen solutions was decreased, no specific adsorption of antibodies could be detected for concentrations smaller than 10^{-6} to 10^{-7}g/ml, whether the slide was introduced wet or dry into the antiserum solution.

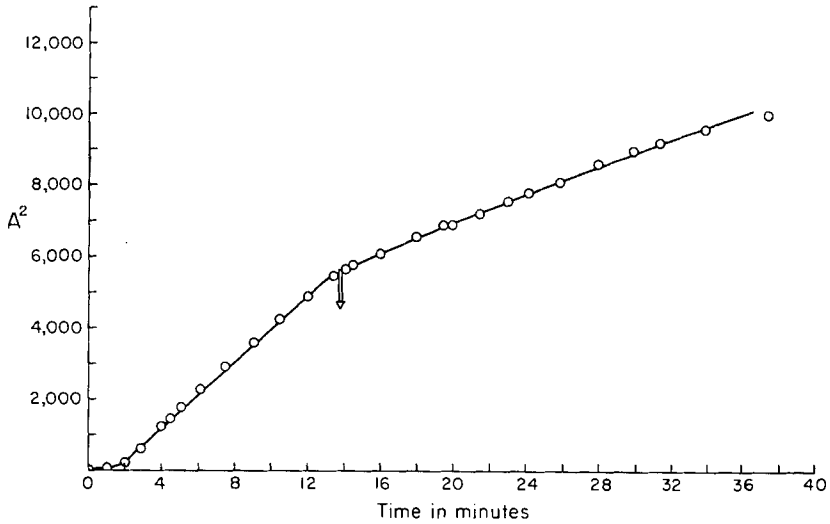

Fig. 2. *Data of curve* a *of Fig. 1, when the square of the thickness* ($Å^2$) *is plotted against time*

The analysis of the curves of the rates of adsorption, *i.e.* thickness adsorbed *vs.* time, showed that the square of the thickness was a linear function of time. After a certain thickness had been adsorbed, a break occurred in this linear relationship which was followed by another linear segment with a less steep slope; more than one break could be observed depending on the system investigated. This is illustrated in Fig. 2 and Fig. 3, where the square of the thickness, $Å^2$, is plotted against time. Fig. 2 corresponds to curve *a* of Fig. 1, and Fig. 3 to curve *b* of Fig. 1. No break appears in Fig. 3, because the thickness at which it takes place had not yet been attained. The linear relationship indicates that the rate of adsorption is controlled by a diffusion process and most likely a 'forced' diffusion since, as we have seen, forces other than those due to a concentration gradient had to be assumed to explain the more rapid rate of

adsorption of antibodies on a wet slide. The breaks which occur in the linear relationship $Å^2$ *vs.* time, show that different energy levels are involved in the adsorption process.

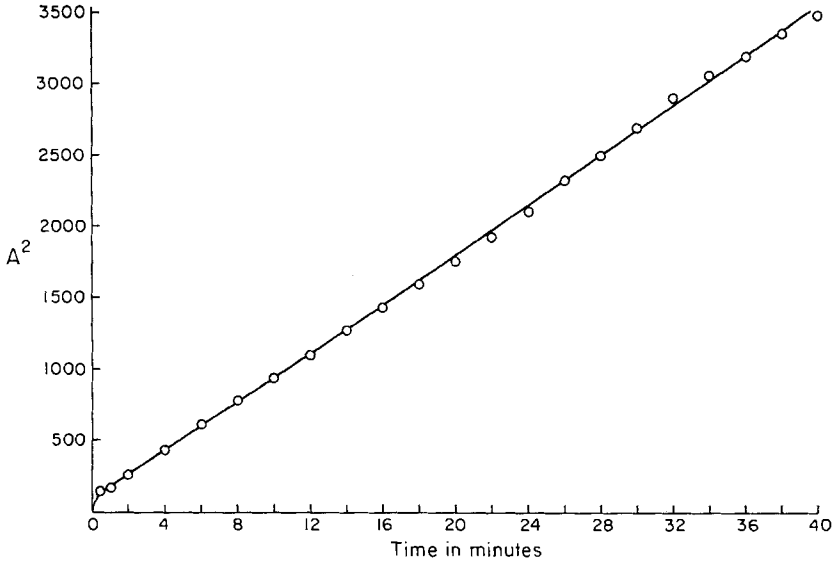

Fig. 3. *Data of curve* b *of Fig. 1, when the square of the thickness* ($Å^2$) *is plotted against time*

As said no specific adsorption of antibodies could be detected with concentrations of the antigen solutions smaller than 10^{-7}g/ml. However, when an electric current was applied during the adsorption from the immune serum, with the slide positively charged, immunologic reactions could be observed with extremely dilute solutions of antigen or of antibodies. Thus a new method called Immunoelectroadsorption was developed which has already found many applications [5].

This method applied to the system polysaccharide from Type VIII *pneumococcus* and the homologous antiserum gave the following results. The pH of the antigenic solution was 2.2. On a slide introduced dry in the antiserum the thickness of the specifically adsorbed layer, that is the thickness adsorbed minus the non-specific thickness observed when antigen was not present in the first adsorption, was approximately a linear function of the logarithm of the concentration of the antigen down to a critical concentration of about 10^{-5} to $10^{-5.5}$g/ml. The non-specific adsorption was under the experimental conditions of the order of 190 to 200 Å depending on the slide. After each determination of the thickness adsorbed from the antiserum on a polysaccharide coated slide, a control experiment was carried out on the same slide on a not coated part.

At the critical concentration of 5×10^{-5}g/ml there was an abrupt change in the slope of specific thickness adsorbed *vs.* log concentration. The specific thickness, which was in the range of 30 Å, then diminished slowly with further decrease in concentration. It dropped rapidly to zero when the limit of sensitivity was reached. The corresponding concentration, called the limiting concentration, was 10^{-13}g/ml in

the experiments summarized in curve *d* of Fig. 4 where the specific thickness adsorbed is plotted against the concentration of the antigen polysaccharide. For high concentrations of antigen in the range of 10^{-3}g/ml, the results were similar to those obtained without current, that is the specific thickness adsorbed on a wet slide was greater than on a dry one as shown by curve *w* of Fig. 4. With increased dilutions of the

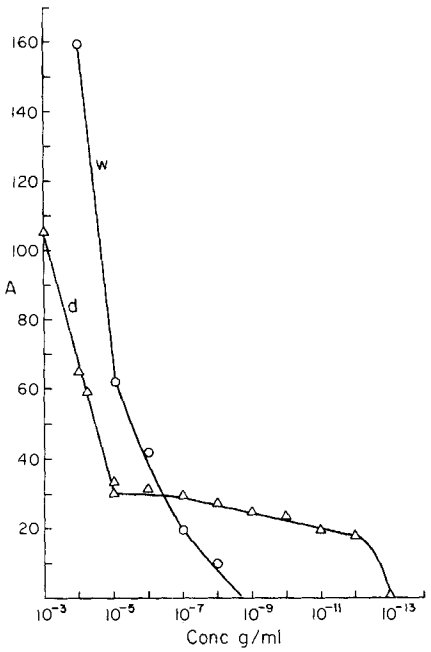

Fig. 4. *Thickness adsorbed in Å units from an antiserum against the polysaccharide from Type VIII* pneumococcus *as a function of the concentration of the solution of the polysaccharide used to coat the slide*

The slide was introduced either wet (curve *w*) or dry (curve *d*) into the antiserum

antigen solutions, a concentration was reached ($\simeq 10^{-6}$g/ml) for which the thickness adsorbed was independent of the condition, wet or dry, of the slide when immersed into the antiserum. For concentrations in antigen lower than 10^{-8}g/ml no adsorption took place on a wet slide, whereas an immunological reaction could still be observed at 10^{-13}g/ml on a dry slide and sometimes down to 10^{-15}g/ml, presumably because the antibody molecules in order to be adsorbed did not have to replace the water molecules surrounding the few antigen molecules present on the slide. It is essential when investigating the effect of the dilution of the antigen on the adsorption of the antibodies as represented in Fig. 4, to use the same type of slides with the same characteristics, especially when working at high dilutions.

The assumption that cooperative phenomena play a major role in these reactions is borne out by the two facts just mentioned.

Firstly, a slide densely coated with antigen can adsorb a thicker layer when introduced wet rather than dry into the antiserum.

Secondly, when the slide is sparsely coated with antigen, is is possible to detect an immunologic reaction at greater dilutions with a dry rather than a wet slide.

When a slide is densely coated with polysaccharide, which can be achieved with a relatively concentrated solution (10^{-3}g/ml), cooperative phenomena between the adsorbed molecules take place with the result that long range specific attractive forces of the *Lifshitz* type develop which are strong enough to pull the antibody molecules through the immobilized layer of water adjacent to the surface of the slide when the slide is wet before immersion. As we have suggested above, the layer of water may act as a specific filter, thus allowing the layer of antibodies to be thicker when adsorbed on a wet, rather than on a dry slide.

The considerable amount of evidence accumulated by us since 1945, indicates that long range interactions can occur in condensed systems of large biological molecules such as antigens and antibodies or protein substrates and enzymes. Interactions could be demonstrated at distances of a few hundred Å and even more in some cases [6] [4]. In 1962, [7] we suggested that such long range interactions could be interpreted in terms of the *Lifshitz* theory which is based on *Van der Waals* forces involving cooperative phenomena between the molecules of the condensed systems. Quite recently, *Ninham* & *Parsegian* [8] have extended the *Lifshitz* theory and shown that in many cases of biological importance, the infra-red adsorption spectra of the reactants are of prime importance and that '*Van der Waals* interactions in hydrocarbon-water systems are peculiarly long range.' Specific interactions in the hundred of angströms could be expected.

It is most remarkable that immunologic reactions could be detected with such dilute solutions of antigen. When the concentration of the polysaccharide was 10^{-12}g/ml, the average thickness attained on a slide was at most 5×10^{-5} Å which would be the thickness if all the polysaccharide molecules present in the 0.5 ml of solution utilized had been adsorbed on the cm² of the immersed part of the slide. As seen on curve *d* of Fig. 4, on such a sparsely coated slide 20 to 30 Å of antibodies could be specifically immobilized. Therefore, what is the mechanism which allows on the slide a ratio by weight of antibodies to antigen of roughly 4×10^5 to 1? Obviously this ratio cannot represent a stoichiometric relationship between antigen and antibodies. It is well known, however, that antigen and antibodies combine in stoichiometric proportions when the reactions are carried out in solution. Entirely new conditions are prevailing when the reactions take place at a liquid-solid interface, with the antigen firmly anchored to the surface. One could assume that the combination antigen-antibody adsorbed on the surface acts as a nucleus around which a large number of antibody molecules could congregate. The energy binding antibody molecules together not directly attached to the antigen, is undoubtedly weak, smaller than KT, hence the aggregates could never be observed in solution where they would be disrupted by Brownian motion. On the other hand, however, additional energy, which could prevent an antibody aggregate from disruption, might come from the energy of adsorption onto the surface. The weak energy of interaction of antibody molecules between themselves would favor the formation of the immobilized aggregates of antibodies, rather than that of other non-specific proteins of the serum. Excellent experimental evidence shows that the immobilization of a large number of antibody molecules around a single antigen molecule is quite dependent on a well

defined crystalline pattern of the metallic layer upon which the reaction takes place [9]. We have found that some slides, which we shall call active, are capable of detecting an immunologic reaction with solutions of antigen at very low concentrations, whereas other slides, called inactive, coated by evaporation with chromium apparently under the same conditions, did not permit the determination of immunologic reactions at concentrations of polysaccharide smaller than 10^{-7}g/ml. Electron diffraction patterns obtained from the surface of the slides revealed that the surface must consist of partially oriented crystallites over 50 Å in diameter for the slides to be active. The surface of an inactive slide is amorphous. However, slides were obtained which exhibited a well defined crystalline pattern but which were inactive for an immunoelectroadsorption assay. In other words, a relatively good crystallinity of the metallic layer is a necessary but not a sufficient condition. The activity of the slide could be cut down by five orders of magnitude if the slide was submitted to a magnetic field of a few thousand gauss for one minute with the lines of force parallel to the surface of the slide. It seems quite probable that in the antibody complex the molecules are similarly oriented in a pseudocrystalline pattern, which is made possible by the regular array of the underlying chromium atoms. The metallic array necessary to provide an 'active' slide does not seem to be specific for carrying one particular immunologic reaction. A slide which was active to determine the immunologic reaction between polysaccharide and antibody at very low concentrations of antigen was also active to determine the immunologic reaction between the antigen obtained from the worms causing schistosomiasis and homologous antibodies present in the sera of patients suffering from this disease. *Vice versa*, an inactive slide was inactive for both of these immunologic systems.

Dilution of the antiserum. In order to observe the large difference in the adsorption from an antiserum between a slide kept either wet or dry after the polysaccharide adsorption, it is essential that the antiserum be diluted at most 1/10 in veronal buffer. At higher dilutions these differences become smaller.

We determined the limiting dilution of the antiserum at which the immunologic reaction ceased to be observed, on slides fully coated with polysaccharide from a solution containing 10^{-3}g/ml of polysaccharide from Type III *pneumococcus*. No current was used during the adsorption period of this antigen which lasted one minute. The antiserum adsorption lasted 2 min, with the slide positively charged as usual. Specific adsorptions of 26 Å, 17 Å and 10 Å were observed on slides treated with a rabbit homologous antiserum diluted 1/4,000, 1/40,000, 1/400,000 respectively. Such specific adsorptions could not be obtained without current. The specific thickness of 10 Å adsorbed at a dilution of 1/400,000 corresponds roughly to the expected thickness if nearly all of the antibodies contained in the 0.5 ml of solution used had been adsorbed ($\simeq 1.5 \times 10^{-7}$g) on the slide.

Conclusions. – Long range interaction between antigen and antibodies due to cooperative phenomena taking place in a condensed system of antigen material, is the most interesting result presented in this article. An immunologic reaction carried out at interfaces shows that a very large number of antibody molecules can be immobilized by a single antigen molecule. This comes as a surprise to immunologists accustomed to deal with interactions performed in an aqueous phase. The results show

that stoichiometric relationships between antigen and antibodies are not sufficient to explain immunologic reactions taking place at a solid interface, where aggregates are obtained which contain a few antigen molecules and a large number of antibodies. Such assemblies might be called Berthollides.

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124. The Structure of the Ferrocenyl-methyl Cation

Preliminary Communication[1])

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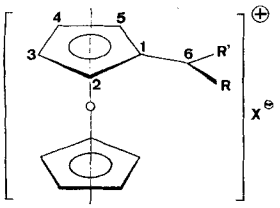
(11. III. 71)

Summary. Using a modified Extended *Hückel* (S.C.C.) procedure the structure of the ferrocenyl-methyl cation has been calculated. The result is a structure (V) with about equal overlap population between all Fe—C bonds. The methylene group is bent towards the iron atom and both rings are tilted.

The best demonstration of the electron releasing effect of the ferrocenyl group is the isolation of stable salts like I [1e]. The diferrocenyl-methyl cation (I, R = ferrocenyl, R' = H) can be isolated as fluoroborate (X = BF_4) and recrystallized from methanol. The NMR. spectra of such ions (I) suggest that the molecule possesses for R = R' a plane of symmetry through the atoms 1 and 6 [1c] [2].

The exact structures of such ions are not yet known and still the subject of some debate [1]. This uncertainty is mostly due to differences in the interpretation of



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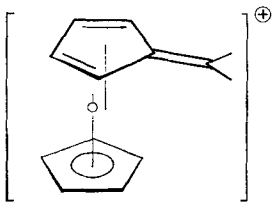

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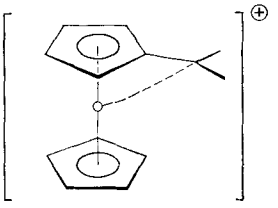

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[1]) A full paper will be published elsewhere.