

DIRECT POLAROGRAPHIC DETERMINATION OF ANTIGEN-ANTIBODY INTERACTION

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Two methods for polarographic determination of the interaction of antigens with their corresponding antibodies were developed recently. The first method, the serographic reaction (Podroužek, 1) is based on the measurement of the stability change of the immune system resulting from antigen-antibody interaction. The stability of the immune system is evaluated by partial standard deproteinization with dilute sulfosalicylic acid. The difference in the deproteinizing effect, measured polarographically in the sample supernatant in ammoniacal hexamokobaltichlorid buffer, represents the stability change of the system which in turn is dependent on the combination of antigen with antibody. The disadvantage of this method is the necessity of diluting the serum before deproteinization, and the deproteinization itself, which can destroy the original equilibrium state of the antigen-antibody system.

In the second method, developed by Breier & Radcliff (Breier & Radcliff (2)), the interaction of antigen with antibody is measured by polarographic determination of antigen concentration. This method is necessarily limited to antigens labelled with polarographically active groups (azo-antigens). The antigen-antibody combination is indicated by the decrease of the polarographic effect of labelled antigen at equivalence point.

Both the above mentioned technics have certain drawbacks that make them unsuitable for practical application. In the serographic reaction—apart of the possibility of disturbing

the established equilibrium—there is required a certain experience and technical skill. The other method is limited to antigens theoretically important but without any practical applicability.

A polarographic method of general applicability for the determination of immune systems combination must suit to several requirements. It must be independent of antigens employed and analytically it must determine the changes produced in the antibody particles as a result of combination with antigen. The antibody is to be polarographed in its native state and the immune system in the equilibrium state, i.e. without additional changes in temperature, nature of electrolytes, changes in the ionic strength, or hydrionic activity of the medium. The method must be as simple as possible without complicated manipulations which could introduce into experimental data errors unaccounted for.

These requirements may be satisfied by making use of the catalytic polarographic praenatrium wave of proteins (Herles and Vančura, 3). However, this wave cannot be evaluated in its original form under conditions of biological isotony and isohydry. By a suitable selection of polarographic derivation equipment (especially of the derivation resistance) it is possible to transform this wave into a smooth, well-reproducible and exactly measurable polarographic curve (Podroužek, Podroužek and Liška, 4).

Protein micelles of blood serum possess a rather considerable buffering capacity. Therefore it is sufficient for obtaining the praenatrium wave by derivation technics to dilute the native serum with 1 per cent solution of NaCl in the ratio 1:20. The catalytic polarographic reaction then proceeds under conditions approaching physiological conditions without any addition of heterogeneous compounds.

The use of the buffering capacity of serum simultaneously with the modified derivation circuit thus permits the direct polarography of the analyzed immune system, without any accessory procedures which could cause disturbances in the established equilibrium state of the immune system.

Derivation resistance: 4000 Ω
 Derivation capacity: 3000 μF
 Time necessary for stabilisation: 3 min.
 Time basis/drop: 25 sec.
 Sensitivity: 1/50.
 Dropping time: 2.9 sec.
 Potential drop: 150 mv.
 The wave begins at 1500 mv.

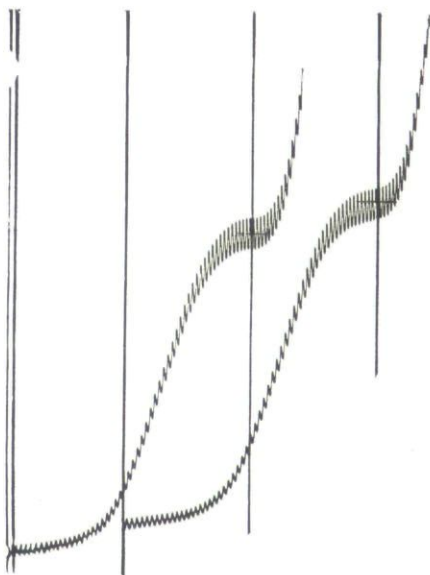


Fig. 1.

The derivated polarographic wave of blood serum, diluted with 1 per cent solution of NaCl in the ration 1:20.

METHODS

In these experiments the pipetting scheme of the serographic reaction was employed (Podroužek, 5). 0.4 ml of immune serum prepared by dilution of native serum with 1 per cent saline (1:20) was added to 0.1 ml of antigen. In control experiments antigen was replaced with 0.1 ml of 1 per cent saline. The reaction mixture was incubated at 18° C for 8 hours. The serum was then transferred into small conical vessels and polarographed. Thus antigen-antibody combination and polarography occurred at the same temperature, consequently the equilibrium state of the immune system was not disturbed by eventual changes in temperature. As 8 hours incubation is sufficient for the swelling equilibrium of the dilute blood serum to become established, the results are not influenced by swelling of the serum proteins during the dilution process (du Noy et al., 6).

The derivation equipment of the polarograph consisted of a capacity of 3,000 μ F combined with the resistance of 4,000 Ω . The value of the derivation resistance was estimated empirically for the employed resistance of the shunt for sensitivity regulation and the dropping time of ca. 3 sec. The potential drop was 150mV for one coil and the sensitivity 1/50 for a galvanometer of the sensitivity 2×10^{-9} A/mm. The time basis of derivation was 25 sec./10 mm. The interval of the dropping electrode was 2.9 sec. The time interval necessary for charging capacities in the derivation circuit was 180 sec. A silver anode was employed as reference electrode; this was a silver wire provided at one end with a silver plate (the wire was fused into a pearl, which was smote into a plate and polished). Before beginning the experiment the anode was cleaned with a metall-cleaning mixture and after each measurement it was cleaned mechanically of adhering reaction products (Fig. 1). The polarographic effect was evaluated as the vertical distance of the abscisse from the top of the wave; a line was drawn through centres of oscillations at the top of the wave and the distance between this line and the abscisse measured. The results were expressed as the wave quotient, i.e. the ratio of the wave height of the sample as compared to the untreated control. A positive result is indicated by a wave quotient higher or lower than 100.

The immune sera used in these experiments were obtained by immunization of rabbits with corresponding antigens. No antiseptics were added, as addition of the latter may cause disturbances in the derivation curves of proteins. Bacterial suspensions employed as antigens were thoroughly washed in the centrifuge with 1 per cent saline. The density of the suspension was determined turbidimetrically. In all experiments the glassware and solutions were sterile, and all procedures were performed aseptically.

RESULTS

In these experiments rabbit antiserum against *S.typhi* was employed. The antigen was diluted in a semidecimal arithmetic

TABLE I
The change of score quotient with the dilution of antigen at constant level of antibodies.

[illegible]

sequence. A similar experiment was carried out with antiserum against *Sh.Boydii*. Table 1 illustrates the results. From these experiments it is evident that the antigen-antibody combination produces a change in the polarographic activity of the dilute serum. The following experiment was designed to establish whether only changes of the polarographic activity of serum proteins are involved in the total polarographic effect or the changes in their buffering capacity as well.

One part of the immune system after combination with antigen was directly polarographed, while the other part was diluted (1:100) with isotonic Coca's phosphate buffer before polarographing. It was supposed that the buffer would compensate eventual changes in the buffering capacity of the immune system. The influence of additional dilution of the system upon the equilibrium cannot be ruled out, nevertheless one can conclude from the results obtained whether the cause of the polarographic activity change of serum proteins is the antigen-antibody interaction or whether the effect of the change in the buffering capacity of the system prevails. The result of this experiment was as follows (table 2).

TABLE 2
The dependence of wave quotient of buffering capacity.
serum dilution:

	With 1 % NaCl	With Coca phosphate solution
Wave quotient	108	132
	106	124

It is evident that the polarographic effect of the stabilized immune system is increased by dilution. However, an increased polarographic effect was observed even with systems in which the antigen-antibody reaction took place in a buffer of higher buffering capacity. It may be concluded therefrom that the change in the polarographic activity of immune systems resulted from the change of the polarographic activity of serum proteins. This change is dependent upon antigen-antibody proportions and increases in the prozone region where a low

concentration of antigen is combined with higher concentrations of antibody. It may be supposed that the polarographic activity change is in some way related to the type of linkage, but a positive polarographic effect, i.e. the increase of the wave quotient certainly is to be expected in the prozone region, and we shall refer further to this phenomenon as to polarographic prozone reaction.

In the above mentioned experiments the concentration of the antigen varied, while the antibody concentration was maintained constant. As some discrepancy may be expected between the alpha- and beta-procedures we have carried out the reaction conversely with a constant amount of antigen and variable concentrations of antibody. To avoid the necessity of diluting the immune sera with a normal rabbit serum, an immune serum obtained during immunization of rabbits with O-antigen of *S. typhi* was employed (table 3). From these experiments it is apparent that the result of the prozone reaction is not dependent upon a particular serological procedure (alpha or beta).

TABLE 3

The change of wave quotient with the change of antibody concentration (active immunisation) and constant level of antigen (Salm. typhi, IX, XII).

Days	1	3	6	9	12
Wave quotient	101	98	106	107	109
Neg. control	100	100	100	100	100

TABLE 4

The prozone polarographic reaction in the precipitation system.

Antigen: heated human milk

Dilution of antigen	1/50	1/100	1/500	1/1000
Wave quotient: exper.	108	105	102	100
	106	104	101	100
Negat. control	100	100	100	100

In experiments with precipitin systems an anticasein rabbit serum was utilized which was obtained from rabbits immunized with centrifuged, heated human milk. The controls were negative rabbit sera. The evidence from these experiments

shows (table 4) that the polarographic prozone reaction is not limited to a special immunological system.

To ascertain at least approximatively which component of the immune serum is active in the polarographic prozone reaction use was made of filter paper electrophoresis. The anti-serum against *Brucella abortus* was fractionned electrophoretically. Unstained stripes were compared with stained ones and cut into albumin and globulin fractions; these were eluted separately in 1 per cent saline. The quantity of saline used was such as to give a final concentration of globulin or albumin in the solution approximately equal to that of the serum. The eluates were combined with the respective antigen and the reaction measured polarographically. The results obtained cannot be compared in this case quantitatively, as the eluates contain variable amounts of the veronal buffer. However, there is an important qualitative difference between the two fractions: the globulins have a positive prozone reaction while the albumins reacted negatively with various dilutions of the antigen. It may be inferred from this that the active component in the polarographic reaction is the globulin fraction (table 5).

TABLE 5

The prozone polarographic reaction in albumin and globulin fraction.

Dil. of antigen ...	Albumin		Globulin		Whole serum	
	1/100	1/1000	1/100	1/1000	1/100	1/1000
Wave quotient ...	100	100	105	102	102	100
Negat. control	100	100	100	100	100	100

In the case of polarographic prozone reaction the rate of establishment of the equilibrium state is a function of two different reactions. First, there is a swelling pressure equilibrium of diluted blood proteins. Further it is dependent on the reaction velocity of antigen-antibody combination. When the swelling equilibrium is eliminated by using for the polarographic reaction a serum already diluted and equilibrated, the following results are obtained (table 6). A positive polarographic prozone reaction takes place almost immediately.

TABLE 6
The prozone polarographic reaction in diluted serum.

	Swelling equilibrium		
	Previously stabilised	Non-stabilised	
		Immediately	After 24 hours
	108	102	108
Wave quotient	106	101	106
	104	103	104

The primary combination of antigen with antibody is an exothermic reaction, while the secondary reaction, i.e. the result of the primary combination, is endothermic. The change in the polarographic activity of the immune serum is a function of the established equilibrium; consequently it is possible by changing the reaction temperatures for the same immune system to determine the reaction heat of the primary specific combination. The prozone polarographic reaction was accomplished at various temperatures under otherwise equal conditions. The immune serum was combined with the antigen and polarographed at the same temperature (table 7). According to our results the polarographic reaction has a negative temperature coefficient and consequently it is exothermic. It may be assumed that in the polarographic prozone reaction the primary reaction of the components of a given immune system is determined. Further support for this conclusion is provided by the fact that the polarographic reaction takes place almost instantaneously.

TABLE 7
The change of prozone polarographical reaction with temperature.

Temperature	18°	25°	30°	37°	40°
Wave quotient	110	108	106	105	104

DISCUSSION

Combination of antigens with the corresponding antibodies results in a change of the polarographic activity of serum globulins, probably of antibodies of globulin nature. This activity change is a function of concentration ratio of the reacting compounds. In the neighbourhood of the equivalence point the polarographic activity decreases. In the prozone region, i.e. in the region of antibody excess, the polarographic activity increases until a maximum is reached; by further increase of antibody concentration the activity begins to fall until its original value is attained. The polarographic prozone reaction is a reaction of globulins. As the albumin is very active polarographically its activity appears on polarographic curves as a constant fraction of the variable globulin activity. The changes in the polarographic activity of the antibody are therefore apparently small as contrasted to the magnitude of the albumin component. The use of dilute native serum as the buffering hydrogen ions donor shifts the reaction to the rising flat part of the Langmuir adsorption isotherm. This is another reason for the polarographic effects not fully displaying themselves. However, if the requirement of the native state of the proteins studied is to be respected, it is experimentally impossible to avoid these undesirable effects. The change of the polarographic activity of the antibody as a result of combination with antigen suggests a possible relation between the changes in the polarographically active groups of the antibody and their hydrophil properties. The hydrogen donors of the polarographically active groups are hydrophil; there might exist a relationship between the change in the hydrophil properties of the antibody and changes in its degree of dispersion resulting from the combination with antigen, in various proportions of the reacting components.

We have noticed that the polarographic prozone reaction is an exothermic process; therefore it may be assumed that it is the result of the primary combination of the reacting components. Because this primary reaction is a monovalent reac-

tion, which takes place irrespectively whether the secondary (lattice-) reaction occurs or not, it appears that the polarographic prozone reaction is a monophasic, monovalent process. It is therefore not limited to common bivalent types of antibodies, but it can be applied as well to monovalent, incomplete antibodies. The antibody amount as determined by the polarographic reaction consequently represents the total amount of all antibodies of the same specificity present.

There exists a possibility for practical utilization of the polarographic reaction for detection and determination of such types of antibodies, to which usual serological methods cannot be successfully applied.

SUMMARY

The interaction of antigen with the corresponding antibody results in a change of polarographic activity of the antibody. This change is caused by the primary combination and can be evaluated by derivation polarography of blood serum.

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