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CORRELATION BETWEEN THE ADSORPTION OF DIPHTHERIA TOXOID AND OF ALIZARIN BY ALUMINUM OXIDE HYDRATE GELS *

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The adsorption of diphtheria toxoid is used to estimate the adsorptive power of Schmidt's aluminum hydroxide gel (1) for the preparation of foot-and-mouth disease vaccine (2). This method is open to criticism, because it is known that anions such as phosphate, borate, carbonate and citrate can inhibit the adsorption of diphtheria toxoid on aluminum hydroxide gel and the toxoid used for the measurement of the adsorptive power is usually dissolved in phosphate buffer (2, 3, 4, 5). Adsorption of Congo Red has also been suggested for the same purpose (6), but its use has been criticized because it is not directly correlated to the adsorption of foot-and-mouth disease virus (5).

This report describes the results of experiments showing that the amount of diphtheria toxoid adsorbed by aluminum oxide hydrate gels of different crystalline structures in presence of chloride ion and in absence of phosphate ion can be correlated to the amount of alizarin adsorbed by the same gels.

MATERIALS AND METHODS

Toxoid. — Prepared according to the methods of the New York State Department of Health (7), the toxoid was precipitated at pH 3.2 and dissolved in the minimum amount of either M/15 phosphate or glycine-potassium acetate buffer of pH 7.2 (7a), and used without separation of the iron porphyrin. The titer of the toxoid was determined by the precipitation method of Ramon (8) and expressed in number of Lf units per me of solution.

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Aluminum oxide hydrate gels. — The preparation of the aluminum oxide hydrate gels used in this study was described in previous papers (9). All gels were freed from soluble salts by dialysis in cellophane bags against distilled water for 2 days. The aluminum content of the gels was determined by the 8-hydroxyquinoleine method (10) and expressed as Al_2O_3, in view of the different water content of the gels. The gels used in the present study were the following: I) pure *Bayerite* gel, from amalgamated aluminum, constituted by Bayerite somatoids (9); II) Willstaetter's *C-gamma* gel, constituted by Bayerite somatoids and Gibbsite hexagonal prisms and platelets (9); III) Willstaetter's "*new b*" gel, from aluminum chloride and ammonium hydroxide, constituted by Bayerite somatoids and Gibbsite hexagonal prisms and platelets; IV) aged *Gibbsite* gel, from aluminum chloride and ammonium hydroxide, constituted by hexagonal prisms and platelets; V) *Boehmite* gel, from amalgamated aluminum, constituted by spherical particles and irregular plates (9); VI) *Schmidt's* gel, constituted by Boehmite fibrils (9, 11); VII) *amorphous aluminum hydroxide* gel, constituted by spherical particles (9); the amorphous aluminum hydroxide was precipitated inside the toxoid solution, previously alkalinized by ammonium hydroxide, by adding aluminum chloride of adequate concentration to adjust the pH to 7.2 and to produce the desired amount of $Al(OH)_3$. Before the adsorption of toxoid the pH of the gels was between 6.5 and 7.0. The particle size, shape and structure of these gels were determined by electron microscopy and by X-ray or electron diffraction, as described in previous papers (9). In this paper, these gels will be referred to under the general denomination of aluminum hydroxide gels.

Adsorption of toxoid. — Diphtheria toxoid solution (400 Lf/ml) was diluted with either M/15 phosphate buffer or 0.85% sodium chloride solution in order to obtain increasing concentrations of toxoid. 1 ml of the gel, containing 20 mg of Al_2O_3, was added to 19 ml of each of these solutions placed in centrifuge tubes, and the mixture thoroughly homogenized by agitation. The tubes were left at room temperature for 24 hours and the precipitate was separated by centrifugation. The toxoid titer was determined in aliquots of the solution before and after adsorption; the amount of toxoid adsorbed was calculated by difference and checked by titration of an eluate of the precipitate obtained with sodium citrate solution. The pH of the toxoid solutions was measured before and after adsorption; it was, in general, between 6.8 and 7.2.

Adsorption of alizarin. — The method for measuring the adsorption of alizarin was a modification of the method used by Weiser to follow the decrease of surface area with aging of aluminum hydroxide gel obtained from hydro-

lysis of amalgamated aluminum (12). The modification was made in order to follow a similar technique to that used for the adsorption of toxoid. A standard M/200 solution of sodium alizarinate was prepared by dissolving purified Merck's alizarin in normal sodium hydroxide and diluting with distilled water to obtain different concentrations of alizarinate; 1 ml of the gel was added to 9 ml of these solutions, the mixture homogenized and centrifuged after one hour. The alizarin content of the supernatant fluid was determined by colorimetry and the amount adsorbed calculated by difference.

RESULTS AND DISCUSSION

Results of the adsorption and the elution of toxoid adsorbed on Boehmite gel are listed in Table I. These data refer to toxoid dissolved in glycine-potassium acetate buffer and diluted with sodium chloride solution.

TABLE I

Adsorption of diphtheria toxoid by Boehmite gel

Tube N.°	total amount of toxoid in 20 ml	number of Lf's adsorbed by 20 mg of Al_2O_3	number of Lf's non-adsorbed in 20 ml	number of Lf's eluted from the adsorbate
1	100	100	-0-	100
2	160	160	-0-	120
3	240	240	-0-	180
4	580	220	360	200
5	720	260	560	240
6	1100	200	900	200
7	1600	200	1400	200
8	2200	200	2000	200

These data show that the adsorption of toxoid in sodium chloride solution follows roughly a Langmuir or Freundlich isotherm, in the same way as observed by McLaren in the adsorption of proteins and enzymes on Kaolinite (13). The departure from a Langmuir isotherm is observed in lower concentrations of toxoid, where no Lf units are found in the supernatant. This fact could be interpreted as a chemisorption of the toxoid on the Boehmite gel by means of a process of ion exchange with the hydroxyl ions on the surface of the Boehmite particles and/or primary valence binding to the aluminum ions on the surface of the Boehmite particles, similarly to what happens in the adsorption of proteins on Kaolinite (13). However, it could also be a physical adsorption of the toxoid on the Boehmite particles if the

number of Lf's left in the supernatant is smaller than the experimental error of the precipitation method of Ramon (8). This type of isotherm was found for all gels, with the exception of Bayerite and Willstaetter's C-gamma gels, which adsorbed only a very small amount of toxoid (4).

The maximum adsorption of toxoid is 13 Lf/mg Al_2O_3 for Boehmite. For higher concentrations of toxoid, the amount of adsorption decreases and remains constant at 10 Lf/mg Al_2O_3. This constant value for the adsorption of toxoid for increasing equilibrium concentrations of toxoids agrees with the hypothesis of an ion exchange mechanism for the adsorption of toxoid, but the occurrence of the maximum of adsorption cannot be explained only by this mechanism.

A complete inhibition of adsorption of toxoid was observed in gels of greater particle size in presence of phosphate ion; on gels of smaller particle size, the adsorption of toxoid in presence of phosphate is smaller as compared with the amount of adsorption in presence of sodium chloride. A significant increase in adsorption of toxoid could be observed, if part of the M/15 phosphate buffer used for dilution was substituted by sodium chloride solution. The values of the amount of adsorption of toxoid on Boehmite gel are higher in presence of sodium chloride than in presence of phosphate ion. These differences can neither be due to the pH (13), nor to the ionic strength of the solution, since after adsorption the pH's were always between 6.8 and 7.2, and there were not found any significant differences in adsorption if NaCl solutions of higher concentration were used as diluents (4).

Table II lists the average size of the particles of the seven aluminum oxide hydrate gels, measured by electron microscopy (9); these values are measured in the directions of maximum elongation of the particles, with the exception of Schmidt's gel, in which case the diameter of fibrils is given instead of their length, which is variable (1).

The amounts of toxoid adsorbed by the seven aluminum oxide hydrate gels are listed in Table II; the numbers refer to the constant and the maximum values of adsorption of toxoid in presence of chloride and phosphate ions.

The amount of adsorption of alizarin or of alizarinate ion on aluminum hydroxide follows a chemisorption isotherm (12); the constant maximum value of adsorption corresponding to the saturation of all aluminum ions on the surface of the particles (14) is listed in Table II, in milliequivalents of alizarin per mg of Al_2O_3.

TABLE II

Amount of adsorption of diphtheria toxoid and alizarin by aluminum oxide hydrate gels

aluminum hydroxide gel	average particle size in millimicrons	number of Lf's absorbed per mg Al_2O_3 inpresence of 0.85% NaCl solution	number of Lf's adsorbed per mg Al_2O_3 in presence of M/15 phosphate buffer	number of milliequivalents of alizarin adsorbed per mg Al_2O_3
Bayerite (I).....	2.200	0.25	-0-	2.03×10^{-5}
C-gamma (II).....	1.400	0.75	-0-	0.88×10^{-5}
New b (III).....	450	3	-0-	2.20×10^{-5}
Gibbsite (IV).....	126	7	1.5	2.33×10^{-5}
Boehmite (V).....	75	10-13	1.5	4.04×10^{-5}
Schmidt's (fibrils) (VI)	30 (diameter)	40-45	-0-	4.80×10^{-5}
Amorphous $Al(OH)_3$ (VII).....	60	500-800	20	44.25×10^{-5}

The data listed in the third and fourth column show that phosphate ion inhibits the adsorption of toxoid, probably by blocking the sites of hydroxyl exchange of the aluminum hydroxide gel. Cole and Jackson (15) found that phosphate ion is adsorbed irreversibly on Gibbsite with formation of dihydroxy aluminum dihydrogen phosphate — $Al(OH)_2H_2PO_4$ (variscite); the formation of that aluminum phosphate happens the same way in which phosphate is fixed in soils. If we assume that the adsorption of toxoid occurs by exchange with hydroxyl ions and binding to the aluminum of the colloidal particle through the carboxyl groups of the protein, the effect of phosphate ion is logically explained by the formation of variscite on the surface of the particles, thus blocking the adsorption sites, since only one of the OH-groups of the aluminum oxide hydrates is free on the surface (16). It is also remarkable that the adsorptive power of amorphous $Al(OH)_3$ is greater in comparison with Schmidt's gels, even in presence of phosphate, which can be explained in basis of the higher surface area of this hydroxide. This explanation may also be applied to the results of Holt (17) who obtained adsorption of 22 to 45 Lf's per milligram of aluminum phosphate gel, because the high value for the surface area of this gel may overcome the inhibiting effect of the phosphate ion (*).

From the above findings it is evident that the use of the amount of adsorption of toxoid as a measure of the adsorptive power may give unreliable results if phosphate ion is present, which thus confirms the criticism made by Pyl on this method (5). If the adsorption of toxoid is made in absence of phosphate ion and presence of monovalent anions such as chloride or acetate, the

(*) Measurements show an average particle size of 33 millimicrons in precipitated aluminum phosphate.

amount of toxoid adsorbed is inversely related to the particle size of the aluminum hydroxide gel and therefore constitutes a reliable estimate of its surface area.

The saturation amount of adsorption of toxoid and of alizarin (columns 3 and 5) decreases proportionally to the average particle size (column 1) of the seven aluminum hydroxide gels, as it could be expected from the dependence of surface area on particle size. The amount of adsorption of alizarin, which is also an estimate of the number of free -OH ion per Al ion on the surface of the particles (14), follows the same pattern as toxoid does in absence of phosphate ion in the medium. Figure 1 shows in a log.-log. scale the correlation existing between the adsorptive power of the seven gels for alizarin measured by the constant or saturation values listed in Table II and for toxoid in presence of 0.85% NaCl solution. The adsorption of aliazarin can thus be used in place of Congo Red to estimate the adsorption of foot-and-mouth disease virus on aluminum hydroxide gels, with the advantage that the mechanism of adsorption of alizarin is better known (14). Moreover, the results indicate that the crystalline structure and/or the particle size or surface area of the aluminum hydroxide gels have influence on the adsorptive power for diphtheria toxoid; gels constituted by Bayerite and Gibbsite are less adsorptive, those constituted by Boehmite intermediate, and the amorphous aluminum hydroxide gel is the most adsorptive one. However, the differences may be due to differences in surface area rather than to different crystalline structure of the gels.

SUMMARY

Adsorption of diphtheria toxoid in presence of phosphate ion may give erroneous results as estimates of the adsorptive power or surface area of aluminum oxide hydrate gels of different particle sizes and structures due to the inhibiting action of the phosphate. If the adsorption is made in presence of chloride ion and in absence of phosphate, it gives reliable estimates of the surface area and these values are in direct correlation to the adsorptive power of the same gels for alizarin. The use of adsorption of alizarin in place of Congo Red is suggested for evaluation of the adsorptive power of aluminum oxide hydrate gels for preparation of foot-and-mouth disease vaccine.

RESUMO

A adsorção de toxóide diftérico em presença de ion fosfato fornece resultados errôneos na avaliação do poder adsortivo de hidróxidos de alumínio constituidos de partículas de diferente dimensão e estrutura, devido à ação ini-

bidora do fosfato. Fazendo-se a adsorção em presença de ion cloreto e na ausência de fosfato, obtém-se resultados seguros na avaliação da área de superfície, e êstes valores estão quantitativamente relacionados com o poder adsortivo dos mesmos géis para alizarina. É sugerido o uso da adsorção de alizarina em lugar do Vermelho de Congo para a avaliação do poder adsortivo de géis de hidróxido de alumínio destinados ao preparo de vacina contra aftosa.

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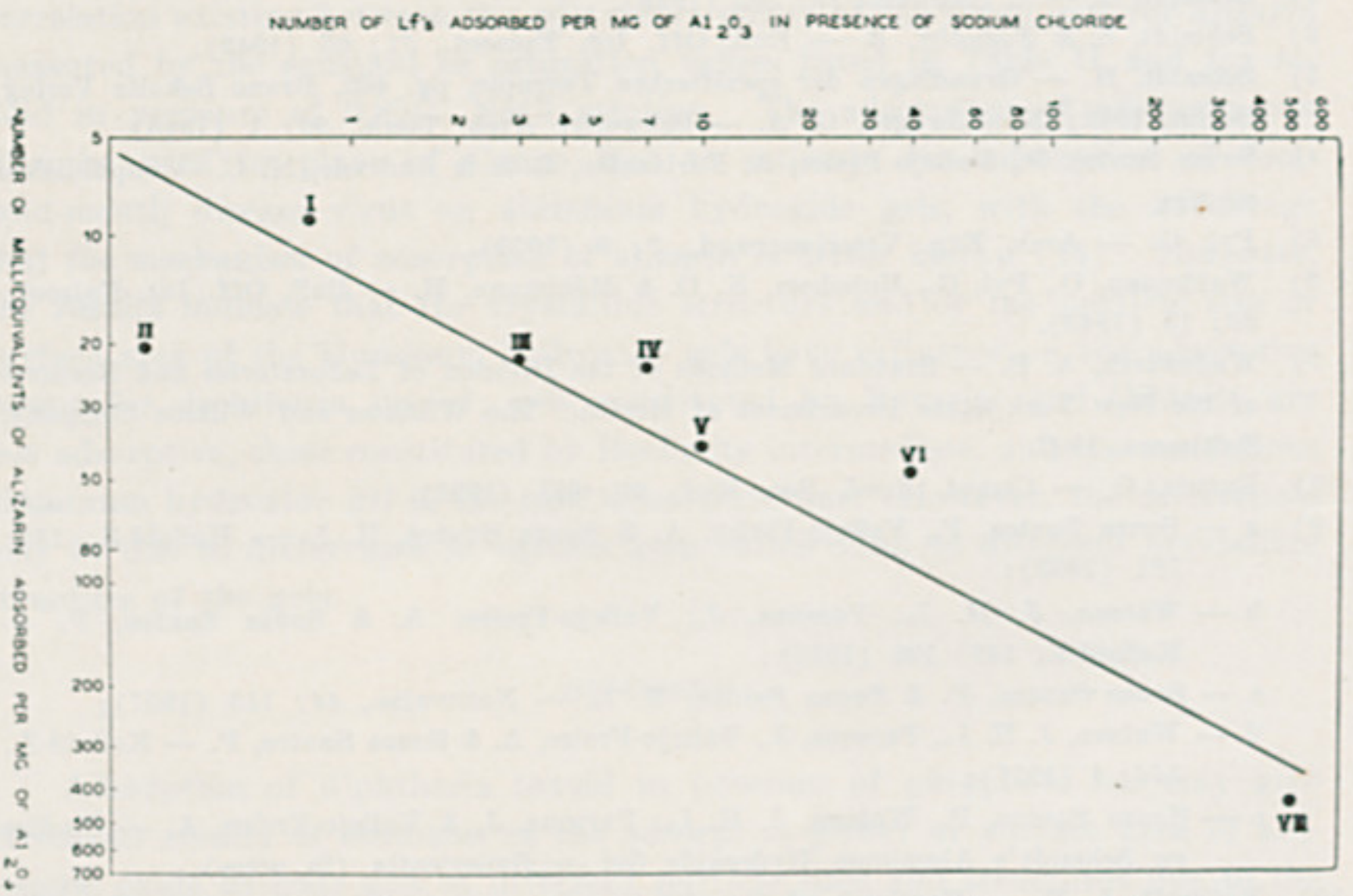

FIGURE 1 — Correlation between the number of units of toxoid and milliequivalents of alizarin adsorbed per miligram of aluminum hydroxide gels of different particle sizes and crystalline structures: the Roman numbers refer to the aluminum hydroxide gels listed in table II.