ADSORPTION OF PROTEINS, ENZYMES AND ANTIBIOTICS BY MONTMORILLONITE

by

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ABSTRACT

Proteins interact with montmorillonite forming mono- and poly-layer complexes. About 20 percent of the protein in a monolayer complex undergoes microbial decomposition and the X-ray pattern remains unchanged, whereas in polylayer complexes the protein undergoes extensive decomposition and the *c*-spacings of these complexes shrink from approximately 30 Å to 12 Å+. Urease is adsorbed completely by H-montmorillonite and only partially by basic montmorillonite. Initial release of urease from the clay is attributed to urea acting as a cation. Subsequently the ammonia evolved by the hydrolysis of urea becomes the active cation. Antibiotic–montmorillonite complexes are classified into three groups. Group I contains strongly basic antibiotics, II amphoteric, and III acid or neutral. The average adsorption of antibiotic in mg per g of clay for each group is: I, 186; II, 307; and III, 9. X-ray diffraction data for groups I and II showed expansion of the *c*-spacing of 4.4 and 7.6 Å, respectively. Bioassays showed no activity for I and appreciable activity for II. The complexes are incapable of diffusing through agar, but the antibiotics must be released first by cationic exchange and then diffuse through the agar.

INTRODUCTION

Soil fertility is due in part to three important constituents of the soil; clays, humus, and microorganisms. It is desirable not only to know the function of each constituent but also how they get along with one another. In general, this is the main objective of this investigation. Here, a few words about humus and microorganisms may be appropriate. Humus is a heterogeneous conglomeration of organic substances derived from plants, animals and microbes. The organic compounds selected for this study are found in the soil and many of them are related chemically by being proteinaceous. The soil swarms with microorganisms. A spoonful of rich soil contains more microorganisms than the total human population on the earth. Under favorable conditions of temperature, moisture and nutrients they multiply very rapidly. As a result of their growth they produce metabolic by-products among which are antibiotic substances. Their metabolism is affected by

https://doi.org/10.1346/CCMN.1960.0090138 Published online by Cambridge University Press

a large variety of enzymes, which they possess. By enzyme action these organisms decompose certain types of organic matter for growth and energy.

In a laboratory study on the effect of clays on the retention of organic matter during humification, it was found that montmorillonite exerted a marked effect in holding carbon, the increase due to addition of 10 percent montmorillonite to sand being nearly twofold in certain cases (Allison, Sherman and Pinck, 1949). In order to elucidate the significance of the above finding, a number of fundamental researches on organic–clay complexes were carried out. The organic compounds studied were two proteins, the enzyme urease, and ten antibiotics. The objectives of this investigation deal with (a) the extent of adsorption and release of the above-named groups of organic compounds by and from montmorillonite, and (b) the physico-chemical properties of the organic–clay complexes. In a few cases, the data will be compared with those of organic–kaolinite complexes.

Since this paper is confined to studies carried on at the Plant Industry Station in Beltsville, Maryland, a review of the literature is omitted, but for those interested in previous work done by others, a few pertinent references are cited: for protein adsorption by montmorillonite (Ensminger and Gieseking, 1939, 1941, 1942; Talibudeen, 1950), for urease in soils (Conrad, 1940, 1942; Hofmann and Schmidt, 1953), and for antibiotics in soils (Gottlieb and Siminoff, 1952; Gottlieb, Siminoff and Martin, 1952; Martin and Gottlieb, 1952, 1955; Siminoff and Gottlieb, 1951).

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Drs. F. E. Allison, S. B. Hendricks, and D. A. Soulides of this organization and to Dr. J. H. Axley of the University of Maryland for helpful suggestions.

RESULTS

Protein–Montmorillonite Complexes

This section will be confined to representative data of the results obtained with protein–montmorillonite complexes. More extensive data and methods of preparation have been published (Pinck and Allison, 1951; Pinck, Dyal and Allison, 1954). Fig. 1 shows comparative rates of decomposition of gelatin present to the extent of 5.9 percent in a complex with montmorillonite, in mixture with it and alone in sand culture. In a period of 4 weeks, the gelatin in the complex, mixture, and gelatin alone decomposed 15, 58 and 80 percent, respectively. The striking difference between the rates of decomposition of the protein in mixture with the clay and protein alone indicate that even under natural conditions protein is adsorbed by montmorillonite. The microbial decomposition of protein in a series of organic–clay complexes is shown in Fig. 2 (gelatin–montmorillonite complexes) and

Fig. 3 (egg albumen–montmorillonite complexes). In complexes containing 10–13 percent protein about 20 percent of the bound protein decomposed, whereas about 80 percent decomposed in those complexes containing 35–54 percent protein. A striking difference in the rates of decompositon of the protein in those complexes containing about 20 percent protein was observed. It should be noted that the data in Figs. 1–3 and Table 1 indicate protein-carbon evolved as CO_2. In addition about 10–15 percent of the decomposed protein goes into microbial growth. Hence the total decomposition is 10–15 percent greater than that reported.

X-ray diffraction data (Table 1) furnish an explanation for the observed variations in the decomposition of the proteins in the complexes. Those complexes having about 10 percent protein and a *c*-spacing of 15 Å cor-

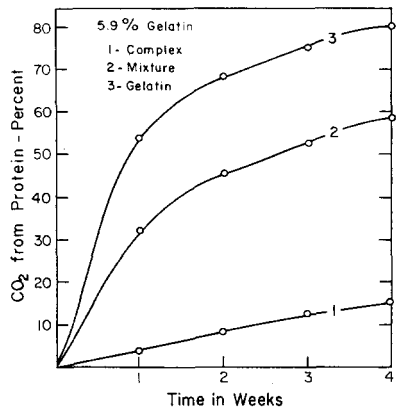

FIGURE 1.—Comparative rates of decomposition of gelatin present to the extent of 5.9 percent in a complex with montmorillonite, in mixture with it, and alone.

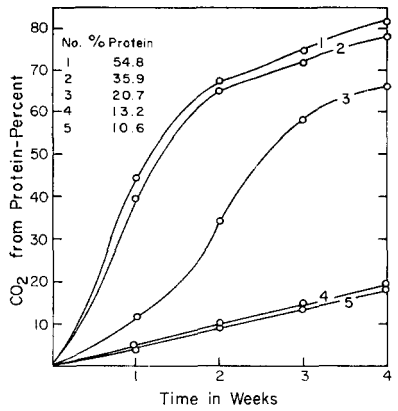

FIGURE 2.—Decomposition of gelatin in gelatin–montmorillonite complexes containing 10.6–54.8 percent protein–in sand culture.

https://doi.org/10.1346/CCMN.1960.0090138 Published online by Cambridge University Press

respond to a monolayer thickness of protein (Talibudeen, 1950). The finding that the *c*-spacing remains practically unaltered after decomposition indicates that the proteolytic enzymes furnished by the soil microorganisms could not penetrate the 4.8 Å spacing as they are larger molecules than the proteins. The 20 percent decomposition that took place in the monolayer of protein was adsorbed on the outer surface and edges which correspond to about 20 percent of the total area of montmorillonite (Hendricks, Nelson and Alexander, 1940). In those complexes containing two or more layers of protein, the latter decomposed to the same extent as where protein alone was added. The commonly observed 12.7 Å *c*-spacing in the residual com-

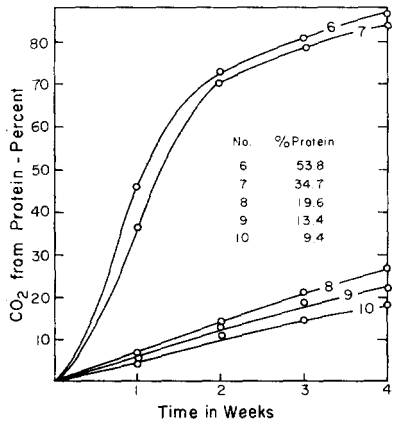

FIGURE 3.—Decomposition of egg albumen in egg albumen–montmorillonite complexes containing 9.4–53.8 percent protein–in sand culture.

plexes (Pinck, Dyal and Allison, 1954) was originally interpreted as an average spacing of complexes with about half of their layers having a *c*-spacing of 15 Å and half having a spacing of 10 Å. Such a mixed-layer complex would not show the regularity of the observed X-ray pattern having

TABLE 1.—X-RAY DIFFRACTION DATA FOR PROTEIN–MONTMORILLONITE COMPLEXES BEFORE AND AFTER DECOMPOSITION BY SOIL MICROORGANISMS FOR 4 WEEKS

Protein in Complex		Protein-carbon Evolved as CO_2	*c*-Spacing	
Kind	Percent	Percent	Before Decomposition (Å)	After Decomposition (Å)
Gelatin	10.6	18.4	15.0	14.6
Egg Albumen	9.4	17.4	15.4	15.0
Gelatin	54.8	82.0	29.2	12.7
Egg Albumen	53.8	86.4	30.0	11.8

a sharp peak corresponding to the observed spacing. It is more likely that the 12 Å+ spacing may be due to the presence of N_4^+H in the crystal lattice resulting from decomposition of the proteins.

Urease–Clay Complexes

The enzyme urease was found to be adsorbed completely by hydrogen montmorillonite, for the supernatant liquid gave a negative test for urease activity, whereas incomplete adsorption was obtained with untreated montmorillonite (pH 8.4) and hydrogen kaolinite (Pinck and Allison, 1961). Data on urease activities of several complexes formed by the addition of 5, 10 and 20 mg samples of commercial urease to 0.5 g samples of H-clays and untreated clays are shown in Table 2. Using urease activity in aqueous

TABLE 2.—ACTIVITY OF UREASE–CLAY COMPLEXES

Kind of Clay	H-Clay			Untreated Clay		
	Urease Additions (mg)					
	5	10	20	5	10	20
	Total ammonia formed (mg)[1]					
Montmorillonite	9.1	16.5	23.5	22.7	39.3	56.6
Kaolinite	17.6	30.3	43.0	19.9	37.2	60.9

[1] Amounts of ammonia formed by 5, 10, and 20 mg urease in water solution were 33.1, 56.2, and 98.2 mg NH_3 respectively.

solution as a standard, it was calculated that H-montmorillonite inactivated about three-fourths of the added urease, and untreated montmorillonite about one-third. The activity of urease adsorbed on H-montmorillonite was about half as great as that adsorbed on H-kaolinite. The activities of urease adsorbed on untreated clays were similar.

An experiment was designed to show whether urease is active in the adsorbed state or whether it has to be released from the clay to become active. The procedure involved the addition of urea to the first supernatant liquid obtained by centrifugation of the urease–H-montmorillonite complex and to the resuspended complex. The suspension was shaken for a short time, and the second supernatant liquid obtained by centrifugation and filtration was divided into two equal aliquots, each of which was titrated with standardized acid for evolved ammonia at different time intervals. The first supernatant liquid gave a negative test for urease activity, whereas the aliquots of the second supernatant gave positive tests increasing with time (Table 3). The results indicate that some urease is released from the H-clay owing to the action of urea as a cation. Although urea is neutral it is known

to form salts with acids. Consequently, in acid solution it behaves as a cation. After the initial release of some urease the evolved ammonia resulting from the hydrolysis of urea raises the pH of the solution, thereby eliminating the cationic action of urease. Ammonia then takes over in releasing more of the bound urease. Such a mechanism explains why the resuspended urease–H-montmorillonite complex exhibits urease activity in contrast to the inactivity of the first supernatant liquid.

TABLE 3.—HYDROLYSIS OF UREA BY UREASE RELEASED FROM UREASE–MONTMORILLONITE COMPLEXES

Time (hr)	Ammonia Formed (mg) in Equal Aliquots of Second Supernatant Liquid	
	a	*b*
0–1.5	2.6	
0–3.5		3.6
1.5–24	7.1	
3.5–24		6.2
24 (total)	9.7	9.8

Antibiotic–Montmorillonite Complexes

Data on the adsorption of ten antibiotics by montmorillonite are shown in Table 4, arranged in three groups according to their reaction characteristics: Group I consists of complexes of strongly basic antibiotics (streptomycin sulfate, dihydrostreptomycin sulfate, neomycin sulfate and kanamycin sulfate), Group II deals with amphoteric antibiotics (chlortetracycline hydrochloride, oxytetracycline hydrochloride and bacitracin) and Group III contains acid (penicillin) and neutral (chloramphenicol and cycloheximide) antibiotics. The amounts of the antibiotics adsorbed are expressed in terms of dry weight of the materials in the forms received. Average values in mg of antibiotics adsorbed by 1 g of montmorillonite in groups I, II and III are 185, 307 and 9, respectively (Pinck, Holton and Allison, 1961).

X-ray diffraction determinations were run on the first two groups of complexes. Studies on Group III were omitted because these antibiotics were adsorbed only to a minor extent. The average expansions (Table 4) of the crystal lattices of Groups I and II were 4.4 and 7.6 Å. On the basis of atom models of representative antibiotics, their amounts adsorbed, and the expansion of the crystal lattices, it was concluded that the complexes of Group I contained monolayers and Group II dilayers of antibiotics.

TABLE 4.—ADSORPTION OF ANTIBIOTICS BY MONTMORILLONITE AND EXPANSION OF c-SPACINGS

Antibiotic in Complex	Mg Antibiotic per g of Clay	Expansion (Å)
Group I—Strongly Basic		
Streptomycin sulfate	209	4.4
Dihydrostreptomycin sulfate	194	4.7
Neomycin sulfate	160	4.3
Kanamycin sulfate	178	4.3
Average	185	4.4
Group II—Amphoteric		
Bacitracin	315	7.6
Chlortetracycline hydrochloride	300	7.5
Oxytetracycline hydrochloride	306	7.7
Average	307	7.6
Group III—Acidic or Neutral		
Penicillin	10	
Chloramphenicol	8	
Cycloheximide	9	
Average	9	

Biological assays for measuring the extent of activity of complexed antibiotics were made by the cylinder-plate method (Grove and Randall, 1955), the results of which are illustrated in Plate 1. Plate 1*A* shows the effect of 20 μg streptomycin in 0.2 ml buffer, in 25 mg montmorillonite complex and in 25 mg kaolinite, each wetted with 0.2 ml buffer. Plate 1*B* shows results with chlortetracycline in solution and complexes. The basic streptomycin–montmorillonite complex failed to show any inhibition of bacterial growth whereas the amphoteric chlortetracycline–montmorillonite showed appreciable inhibition. Experimental evidence was obtained indicating that an antibiotic is first released from the clay by an exchange reaction with the cation of the buffer and then diffuses through the agar (Pinck, Soulides and Allison, 1961).

With the object of determining the smallest quantity of an antibiotic that could be detected in 1 g of clay, bioassays were made on antibiotic–montmorillonite complexes containing minimal quantities of antibiotics. For comparison bioassays were also made on buffer solutions of the antibiotics, and on antibiotic–kaolinite complexes (Table 5). The previously mentioned division of the complexes into groups depending upon the reaction of the antibiotics applies equally well to their bactericidal activities. The montmorillonite complexes of the strongly basic antibiotics failed to show any activity, whereas those containing amphoteric antibiotics gave positive tests. Although kaolinite complexes of Groups I and II gave positive

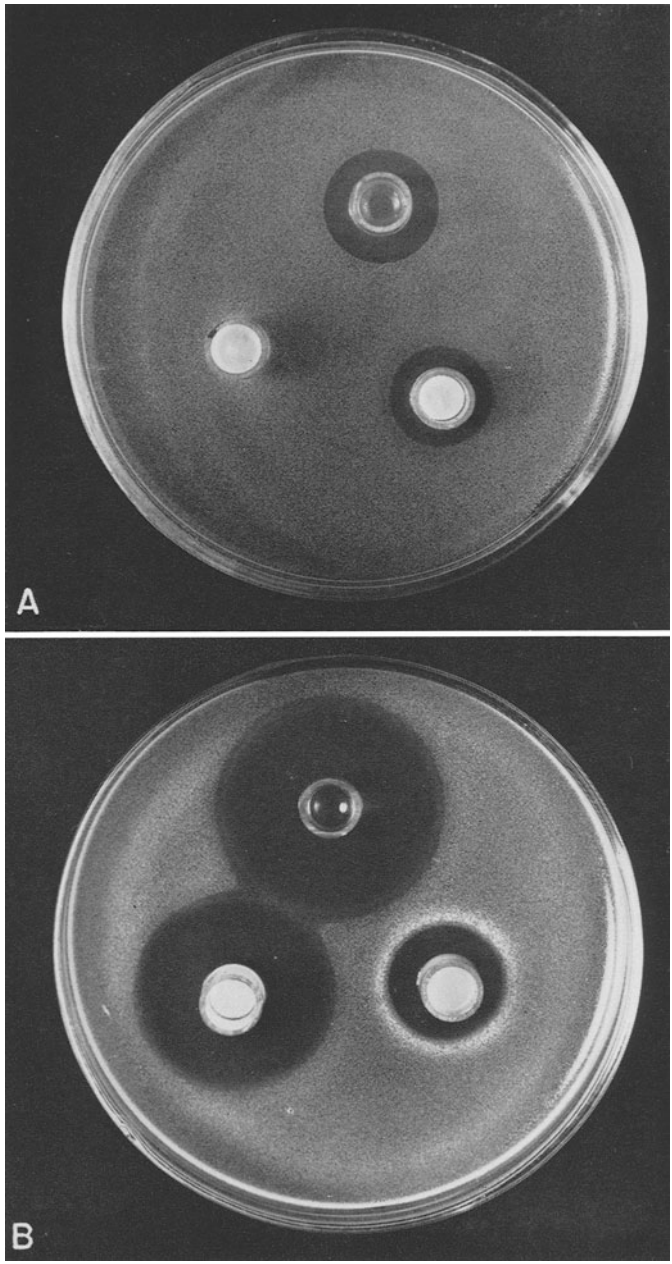

PLATE 1.—(*A*) Activity of streptomycin–clay complexes. Control (top), montmorillonite (left) and kaolinite (right).
(*B*) Activity of chlortetracycline–clay complexes. Control (top), montmorillonite (right) and kaolinite (left).

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tests there was a marked difference in the sensitivity of the two groups when compared with their respective controls. The differences in activities in the kaolinitic and montmorillonitic complexes of the basic antibiotics are attributed to differences in Coulomb forces of the two clay minerals, for montmorillonite has a much greater degree of substitution in the tetrahedral and octahedral layers than does kaolinite.

TABLE 5.—MINIMUM DETECTABLE AMOUNTS OF ANTIBIOTICS RELEASED FROM MONTMORILLONITE AND KAOLINITE COMPLEXES[1]

Clay	Group I—Strongly Basic Antibiotics				Group II—Amphoteric Antibiotics		
	Strepto-mycin	Dihydro-strepto-mycin	Neo-mycin	Kana-mycin	Baci-tracin	Chlortetra-cycline	Oxytetra-cycline
Montmorillonite	—	—	—	—	800	0.5	2.0
Kaolinite	25	25	800	200	1	0.05	0.4
Control	1	1	2.5	0.5	0.25	0.02	0.1

[1] Figures for complexes and controls in μg of antibiotic per g and ml respectively.

DISCUSSIONS AND CONCLUSIONS

The adsorption of proteins and antibiotics by montmorillonite involves a base exchange reaction. The slight degree of adsorption of acidic and neutral antibiotics by montmorillonite in comparison with the extensive adsorption of basic and amphoteric antibiotics and of proteins supports this conclusion.

Monolayers of proteins and antibiotics are more rigidly held to montmorillonite than polylayers. This may be attributed to Coulomb and van der Waals forces (Hendricks, 1941).

The results indicate a common mechanism of release and activity of urease and amphoteric antibiotics complexed with montmorillonite, implying that enzymatic and antibiotic activities take place in aqueous solution and not on clay surfaces,

In decomposition studies of protein– and urease–montmorillonite complexes, the pH rose considerably above the isoelectric points of the organic compounds as a result of the evolution of ammonia, and in the bioassays of the complexed amphoteric antibiotics, the pH was adjusted to a reaction above the isoelectric point by means of buffers. Since amphoteric compounds are dependent upon low pH to maintain their cationic state, any reaction which causes a rise in pH lowers the cationic concentration with a concurrent rise in concentration of anions. Hence the electrostatic forces originally present are greatly weakened, thereby permitting a release of

the organic matter. In the case of the basic antibiotics, a change in pH has no effect on the Coulomb forces; consequently they are not released from the clay.

In conclusion the results of this study show a clear-cut relationship in the behavior of proteins, urease and antibiotics with montmorillonite; they also show how extensively the multifarious functions of the microorganisms affect the adsorption and release of organic matter by and from the clay.

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