Interaction of Hexachlorophene with Human Epidermis I: *In Vivo* Bioelectrometric Study of pH Influence

ROLLAND I. POUST and VICTOR F. SMOLEN

Abstract □ The effects of pH and hexachlorophene on the net fixed-charge density of the colloids composing the epidermal surface were studied *in vivo* employing human subjects. The results are analogous to titration curves of amphoteric macromolecules. The study was performed over a pH range of 1.1–12.3 in the presence and absence of saturated concentrations of hexachlorophene. Both the control and hexachlorophene titration curves were nonhysteretic when initiated at either extreme of the pH range; this finding indicates reversibility of the observed pH and hexachlorophene-induced changes. The presence of hexachlorophene decreased the net fixed-charge density of the tissue surface below pH 5.6. Above pH 5.6, a net increase was consistently observed. Mechanistically, the hexachlorophene-induced alterations in fixed-charge density are hypothesized as resulting from allosterically effected changes in the dissociation constants of ionogenic protein side groups located vicinally to hexachlorophene interaction sites. The bioelectrometric method allowed the study to be performed without causing any injury or discomfort to the subjects. The results of the present study suggest a further utility for the bioelectrometic method in studies of the substantive antibacterial behavior of hexachlorophene on human skin.

Keyphrases □ Hexachlorophene interaction—human epidermis □ Epidermal surface colloids—acid-base binding properties □ Charge density, fixed—human epidermis □ Bioelectrometric study —pH effect, hexachlorophene–human epidermis interaction

Following transient exposure, the substantive effectiveness of a skin antibacterial such as hexachlorophene largely depends upon the extent of its deposition onto the colloids composing the stratum corneum as well as the manner in which it is subsequently available to affect microorganisms. Commonly employed experimental techniques do not always permit the substantive activity of skin antibacterials to be readily evidenced, particularly under conditions of actual product usage. The recovery and quantification of very small amounts of the antibacterial agents are often necessary. Some techniques require the excision of the treated skin and are, therefore, inapplicable for extended and routine use with human subjects. Techniques that involve the disintegration of the skin and fractionation of its constituents are further disadvantaged by the obvious difficulty of distinguishing and correcting for antibacterial bound to the colloid *in vivo* and that which becomes interacted *in vitro* through the activation of binding sites by the *in vitro* treatment of the tissue.

A bioelectrometric method, which has been successfully applied in studies of the interaction of other substances (1–5)—some of which have been phenolic (6)—could conceivably be applied to allow the detection of residual, bound quantities of hexachlorophene on human skin *in vivo* under realistic conditions of product usage, without any chance of injury resulting from the measurements.

The purpose of the present study was to determine the sensitivity of the bioelectrometric method in detecting changes in the net fixed-charge density of the epidermal surface occurring in response to the interaction of hexachlorophene. The interpretation of the results of the investigation was anticipated to provide some mechanistic insight into the nature of observed phenomena.

METHOD

The application of the bioelectrometric method to the study of solute interaction with tissue surfaces is dependent on the detection of solute-induced changes in the net fixed-charge density of the surface colloids relative to suitable controls. The fixed charge originates from the dissociation of counterions from immobile ionogenic groups covalently bonded or otherwise sorbed onto the surface. The magnitude of the fixed-charge density, at any given composition of applied solution, determines the magnitude of the electrical potential difference developing across the colloid–aqueous boundary. The measurement of a potential, E_1, includes this equilibrium phase boundary potential as well as all other potentials developed in the circuit. These extraneous potentials have been found to remain constant for the short period of time required to replace the initial solution in contact with the tissue surface with a dilution of this same solution, followed by the immediate recording of a second potential, E_2.[1] The difference of these two measured potentials ($E_2 - E_1$), termed the dilution potential, E_d, is devoid of extraneous potentials and includes only those potential differences developed between the aqueous medium at the tissue surface and the bulk of the applied solution. This consideration allows the interpretation of E_d in terms of the familiar Donnan equilibrium (7) and its treatment as a diffusion potential (8). Under the conditions of its measurement, E_d is characteristic only of the tissue surface rather than reflecting the properties of the bulk or interior of the tissue phase.

General equations relating the dilution potential and fixed-charge density (f) have been presented in earlier reports (1, 2). Determinations of the fixed-charge density of tissue surfaces under conditions of varying pH resemble and are analogous to pH titration curves of amphoteric macromolecules (9) or insoluble colloids (10, 11) when plotted as a function of pH. The form of these curves is dependent upon the nature of the ionizing groups, the presence of interacting solutes, and the state of aggregation of the colloids composing the tissue surface. The fixed-charge density on tissue surfaces is often quite sensitive to interacting substances, as reflected in alterations in the shapes of titration curves. Such changes can often be interpreted to yield information concerning the extent and nature of the interaction.

Details of the experimental arrangement, preparation of the tissue surface, and precautions concerning how the measurements are to be performed have been reported earlier for human finger epidermis (2). Briefly, the experimental procedure consists of standardizing the condition of the skin by thorough cleansing and allowing the skin to become prehydrated by soaking in 0.15 *N* NaCl for approximately 15 min. This treatment also removes labile, water-soluble, ionogenic materials (12), which would otherwise be elutriated during the measurements. Such losses contribute to uncontrolled variations in the measured potentials. As shown in Fig. 1, the experimental finger and a saturated calomel electrode are placed together into a buffered isoosmotic solution, which may or may not contain the solute whose interaction with the surface is of interest. The reference finger is placed into another beaker containing 0.15 *N* NaCl solution along with another saturated calomel electrode. Neither the reference finger nor the electrode is further

[1] The solutions used for the measurment of E_2 were 10-fold dilutions with regard to all components except the hexachlorophene.

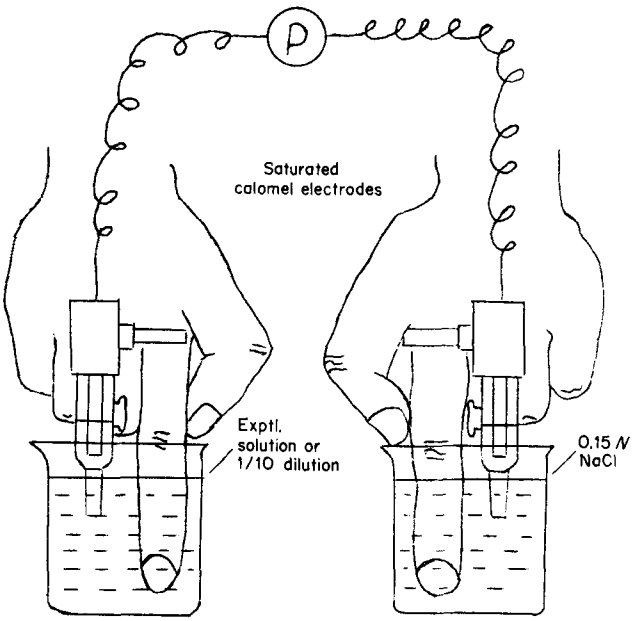


Figure 1—*Diagram of experimental arrangement for the electrometric study of hexachlorophene interaction with human finger epidermis. P = potentiometric device.*

disturbed during the experiment.

The internal circuit between the experimental and reference fingers is completed by the body of the subject. The electrodes in the external circuit are connected to a potentiometric device used for the null point reading of the developed potentials. The potential, E_1, is recorded until its observed variations become less than approximately 0.2 mv./min. When the epidermis is pretreated as previously described, this stability is usually achieved within 1 min. following immersion. Following the measurement of E_1, the finger is removed and immersed in a 1/10 dilution of the same solution; the potential, E_2, is immediately recorded. The finger is then removed from the 10-fold dilution and reimmersed in the previous solution. The potential, E_1, is again measured and compared to the previously determined value to ensure that the extraneous potentials in the circuit have remained constant. Agreement within 1.0 mv. was taken as acceptable. The mean of the two measured values was used to obtain the dilution potential, E_d.

MATERIALS

Corning, miniature, fiber-junction, calomel reference electrodes were used in conjuction with a Heath pH electrometer-recorder. The potentials were read directly from the recorder chart. The potential measurements were performed on three male, human volunteers ranging from 20 to 25 years of age. All reagents were of analytical grade, except for hexachlorophene.[2]

The buffered solutions used for the determination of E_1 were each prepared to an ionic strength of 0.15 and a buffer capacity of 0.0288. This buffer capacity is approximately equivalent to that of a 0.05 *M* phosphate buffer at pH 7.21. The ionic strength is the same as that of isotonic sodium chloride. The compositions of these solutions are listed in Table I. Tenfold dilutions of these solutions, as well as of each of these solutions saturated with hexachlorophene, were also prepared and used in the experiment.

Calculations were performed with the aid of a CDC 6500 digital computer. Calibration curves, relating measured values of the dilution potential to corresponding theoretical values of the fixed-charge density, were constructed with a model 563 Calcomp digital incremental plotter.[3] Representative curves, corresponding to several buffer solutions, are shown in Fig. 2. Figure 3 contains a corresponding nomograph. The verity of the calibration curves was tested experimentally through the *in vitro* measurement of E_d values corresponding to zero net fixed-charge density, *i.e.*, E_d^0 values.

In the absence of fixed charge, the E_d^0 values are tantamount to liquid junction potentials across a free diffusion-type boundary (8). Such boundaries may be conveniently formed using a filter paper bridge between each solution and its 10-fold dilution. However, an error can result in such measurements if a fixed charge resides on the filter paper. To determine whether an error is introduced from this source, diffusion potential measurements were performed with the formation of a liquid junction, between 0.1 and 0.01 *N* KCl solutions, within the filter paper. The measured potential had a mean value of 0.8 mv., indicating that the filter paper was effectively neutral and suitable as a matrix for the formation of free diffusion boundaries. Diffusion potentials measured in this manner have previously been found to agree generally within 1 mv. with values determined using free diffusion boundaries formed within glass capillaries (1).

The averages of six determinations of E_d^0 values are listed in Table II with their corresponding standard deviations. The agreement between experimental and calculated values is most often within 1 mv. and is considered acceptable. The presence of saturated concentrations of hexachlorophene in both the buffers and their 10-fold dilutions does not appear to influence the magnitude of the measured potentials.

To determine the influence of pH on the interaction of hexachlorophene with the epidermal colloids, the fixed-charge density of the tissue surface was determined at each of the 12 pH values de-

Table I—Composition of Buffer Solutions Employed in the Determination of Titration Curves of Tissue Surfaces (Concentrations in Moles/Liter)

pH	Components	Sodium Chloride
1.0	Hydrochloric acid, 0.1	0.05
2.2	Hydrochloric acid, 0.0061	0.1439
2.9	Hydrochloric acid, 0.0013	0.1487
3.7	Sodium acetate, 0.0136; acetic acid, 0.155	0.1364
4.6	Sodium acetate, 0.0211; acetic acid, 0.0304	0.1289
5.5	Sodium acetate, 0.0821; acetic acid, 0.0055	0.0679
6.5	Sodium phosphate, dibasic, 0.0148; sodium phosphate, monobasic, 0.0762	0.0344
7.4	Sodium phosphate, dibasic, 0.0319; sodium phosphate, monobasic, 0.0206	0.0337
8.5	Ammonium hydroxide, 0.015; ammonium chloride, 0.0816	0.0684
10.0	Ammonium hydroxide, 0.0613; ammonium chloride, 0.0104	0.1396
11.5	Sodium hydroxide, 0.00316	0.1438
13.0	Sodium hydroxide, 0.1	0.05

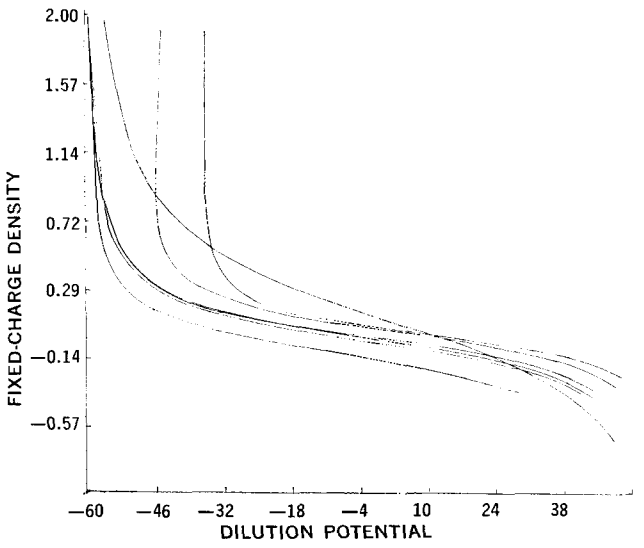


Figure 2—*Plot of calibration curves for several buffered measuring solutions using the digital incremental plotter.*

[2] Supplied by Armour-Dial, Inc., Chicago, Ill.
[3] California Computer Products, Inc., Anaheim, Calif.

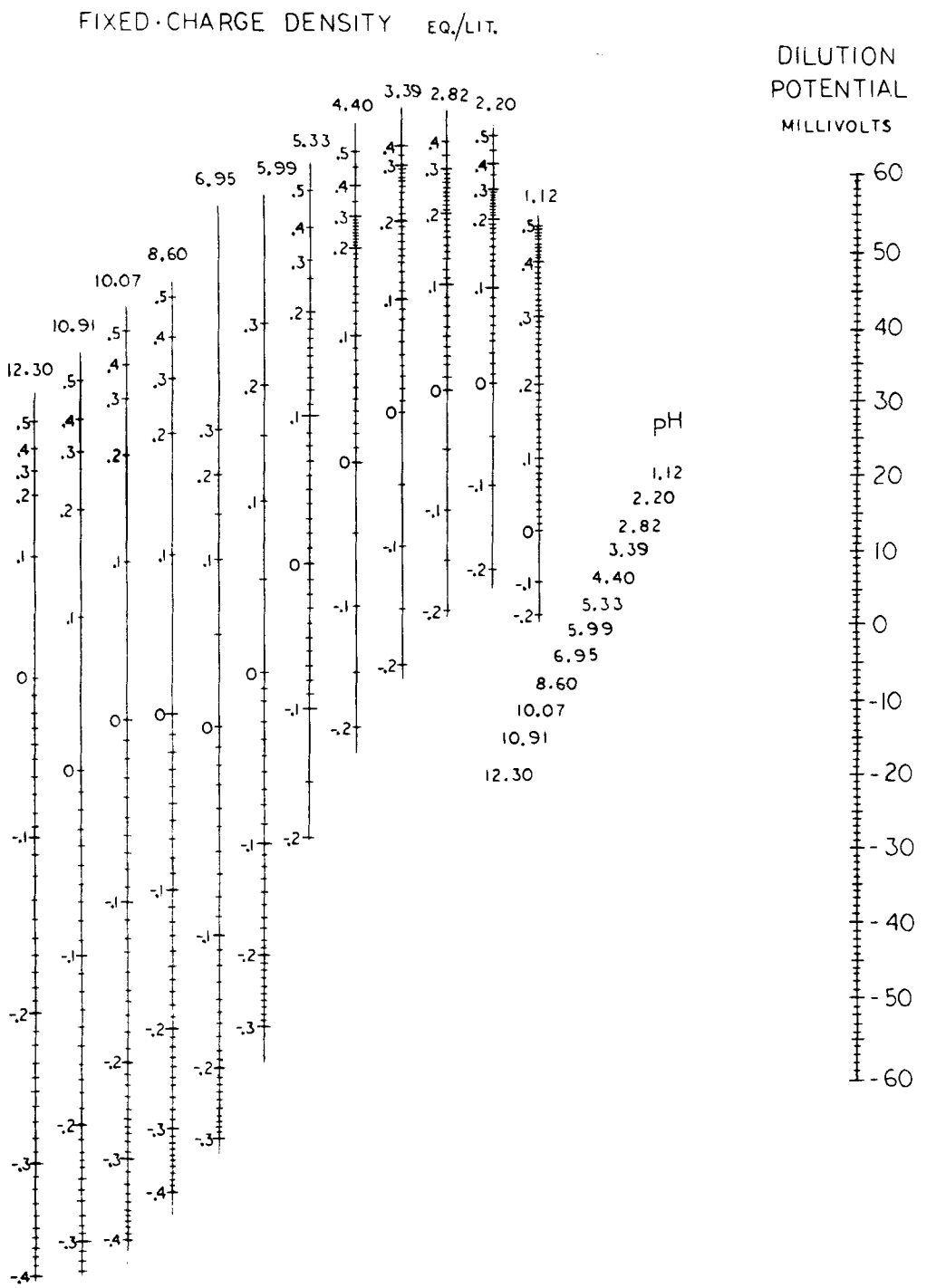


Figure 3—*Alignment Chart relating experimentally observed values of the dilution potential to the corresponding density of fixed charge. To read the fixed-charge density, a straight line is passed to the left from the dilution potential, through the decimal point of the pH of the solution, and intersected with the fixed-charge density scale corresponding to the same solution pH.*

scribed, both in the absence and presence of hexachlorophene. The use of the controls permitted the changes in fixed-charge density due to hexachlorophene interaction to be evidenced. Measurement of E_d values for the control points were made following the attainment of an apparent equilibrium of the solutions with the surface; usually only a few minutes were required. However, the measurements with hexachlorophene-containing solutions were made after 46 min. to allow time for complete interaction with the epidermal surface.

RESULTS AND DISCUSSION

Normal and Hexachlorophene-Displaced Titration Curves—Figure 4 demonstrates the influence of pH on the net fixed-charge density of normal and hexachlorophene-treated human finger epidermis. The results resemble and are analogous to hydrogen-ion titration curves of proteins. A reversibility in the effects of pH and hexachlorophene on the fixed-charge density was indicated by a lack of any systematic differences between titration curves, differing only in that the measurements were initiated at low and high pH. This conclusion was found to be further supported statistically by the results of a paired t test comparison performed between the up-curves and the down-curves; such curves were statistically the same in the case of both the control and hexachlorophene-treated skin. It was, therefore, concluded that the interactions of the solution components with the colloids constituting the epidermal surface are reversible.

Inspection of the curves reveals that, relative to the control curve below approximately pH 5, hexachlorophene induces a net decrease in the density of fixed positive charge on the epidermal surface and causes a small shift in the isoelectric point in the direction of a more acidic pH. In contrast, above the isoelectric point, near pH 5, a hexachlorophene-induced reduction in the fixed anionic charge density of the epidermal surface is observed. It is pertinent to note that, in excess of this pH, the proportion of hexachlorophene [pKa =

Table II—Comparison of Calculated and Experimental Dilution Potentials for Control and Hexachlorophene-Containing Buffer Solutions

pH of Buffer	Calculated	Observed Potentials	
		Plain Buffer	HCP in Buffer
1.12	31.87	31.58 ± 1.63	33.65 ± 1.36
2.20	−5.89	−3.56 ± 0.24	−3.30 ± 0.65
2.82	−12.04	−11.50 ± 1.64	−8.72 ± 0.93
3.39	−11.14	−12.80 ± 2.96	−9.50 ± 1.30
4.40	−10.42	−8.25 ± 0.48	−9.57 ± 1.12
5.33	−3.83	−2.43 ± 0.29	−4.17 ± 0.87
5.99	4.65	6.83 ± 0.23	6.83 ± 0.90
6.95	4.72	6.10 ± 0.34	6.60 ± 0.60
8.60	−5.81	−4.95 ± 0.19	−5.20 ± 1.19
10.07	−11.68	−9.70 ± 0.18	−8.23 ± 1.41
10.91	−13.36	−10.90 ± 0.32	−10.78 ± 0.98
12.30	−31.57	−27.15 ± 0.35	−31.47 ± 1.50

5.4 (12)] existing in solution as anions relative to unionized hexachlorophene increases very rapidly with increased alkalinity. It, therefore, appears that the reduction in net positive surface charge observed at low pH may be reasonably attributed to the results of the interaction of unionized hexachlorophene, while hexachlorophene anions are apparently responsible for the reduction of net negative charge in the less acidic and alkaline pH range. Although an interaction of anions with a negatively charged surface, which results in a reduction of negative charge density, may initially appear to be a rather unique phenomenon, it has previously been observed to occur in similar studies involving phenolic (6) and other anions (2, 4). The interaction of anions with proteins in solution above their isoelectric point has also been commonly reported (13).

Mechanism of Hexachlorophene Effects—Solute-induced changes in fixed-charge density, which result in the displacement of titration curves such as observed for the effects of hexachlorophene, can result from an irreversible loss of titrable groups from the surface or through the masking of their charge. Changes in the fixed-charge density, which might occur from the elutriative loss of charged materials from the skin (14, 15), are minimized by the treatment of the skin performed prior to the measurements. Any further elutriative losses, which could conceivably occur during the measurements, are apparently minimal as indicated by the previously described reversible nature of the effects. Since irreversible chemisorption is obviously also not a plausible mechanism, the hexachlorophene effects must be attributed to a masking of fixed surface charge.

Since at low pH the hexachlorophene is unionized and at high pH the interaction of anions lowers the anionic fixed-charge density, the observed phenomena must occur by a means other than a direct neutralization such as could result from salt binding of the hexachlorophene to the ionogenic fixed surface groups. Since hexachlorophene is a dihydric phenol, its mode of interaction with proteins may be expected to resemble that attributed to phenols in general. Küntzel (16), in describing the action of phenols on collagen, suggested that their interaction with proteins occurs through hydrogen bonding of the phenols with the peptide linkages constituting the protein backbone. The affinity of the interacting molecules for the protein is postulated to result from a polarity induced into their aromatic nuclei by the phenolic hydroxyl group; there results a development of activated CH groups with alternative net positive and negative partial charges. The CH group adjacent to the hydroxyl is postulated as the primary binding site within the molecule.

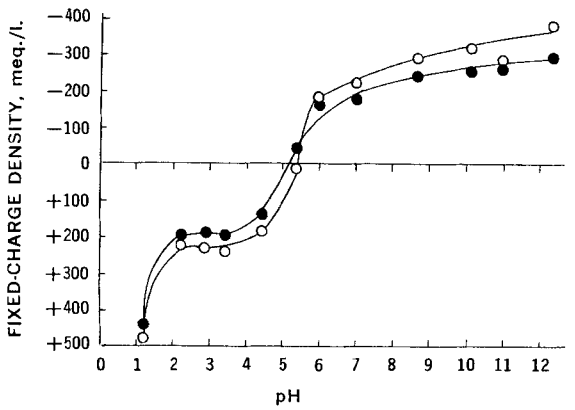


Figure 4—*Control* (○) *and hexachlorophene-displaced* (●) *titration curves of human finger epidermis. Each point is the average of six replicates on one subject.*

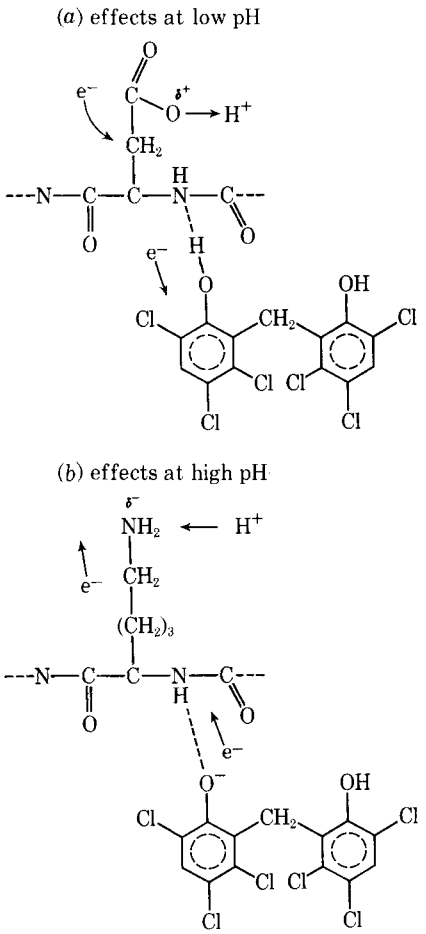
Pankhurst (17), on the other hand, suggested that the binding of phenolic molecules occurs directly by the formation of hydrogen bonds between the phenolic hydroxyl and the peptide nitrogen. Conceivably both sites may be operative and serve to complement one another; however, the direct hydrogen bonding of the hydroxyl appears to be more reasonable in providing the primary mode of interaction (18). In any event, only peptide linkages could occur in sufficient number to provide the binding sites for the extraordinarily large quantity of phenols commonly observed to be sorbed by proteins. That the sorption does not directly involve ionogenic groups is indicated by the mineral acid-binding capacity of collagen, for example, remaining unchanged (18). The degree of dissociation of ionogenic groups located vicinally to hexachlorophene-interacted peptide sites may, however, be influenced indirectly by hexachlorophene binding. Such influences can be propagated through space by direct electrostatic field effects or through the atoms constituting the protein itself through an inductive delocalization of electrons (19). Both mechanisms can operate to alter the acidic pK's of ionizable vicinal groups. The observed changes in fixed-charge density of the epidermal surface, therefore, can be postulated to occur as a consequence of an uptake or release of protons occurring concomitantly with the binding of hexachlorophene with peptide linkages vicinal to the affected ionogenic groups. Electron inductive effects have been shown to be effectively transmitted through as many as five saturated carbon atoms (20). Pauling (21) described the transmission of inductive effects through peptide linkages to be facilitated by the high polarizability, due to the partial resonance, of the peptide amide bond, which has been discerned to possess a transmissivity for inductive effects approximately tantamount to two methylene groups (22). Ling (22) and Szent-Györgi (23) convincingly postulated that such effects can be transmitted through hydrogen bonds to alter the reactivity of groups located in positions neighboring the binding sites of hydrogen bond-forming interactants.

Based on these considerations, the electronic mechanisms operative in the manifest effects of hexachlorophene on the epidermal fixed-charge density can be postulated to occur as diagrammatically described in Scheme I.

The influence of hexachlorophene at low pH must primarily be directed toward the fixed carboxyl groups, which are titrable within the pH range of 2–6 (9). Carboxyl groups are plentiful in the stratum corneum. It has been found (24) that aspartyl and glutamyl residues comprise approximately 21.7% of the weight of keratin, the major proteinaceous constituent of stratum corneum.

At low pH the hydrogen bonding of the electrophilic phenolic hydroxyl with the peptide linkage may be envisaged to induce a withdrawal of delocalizeable electronic charge from the carboxyl group of a vicinal aspartyl or glutamyl side group, resulting in a lowering of the acidic pK of the groups. The accompanying release of protons into the milieu manifests as a reduction in the net positive fixed-charge density of the surface. The high density of carboxyl groups and their separation from the polypeptide backbone by only one or two methylene groups contribute to an expected high probability that such groups may be located within the inductive influence of bound hexachlorophene. The presence of the three electronegative chlorine atoms on the aromatic nuclei of the hexachlorophene molecule may be expected to contribute substantially to its electrophilic inductive efficacy.

At high pH the electrophilic character of the hexachlorophene can be considered as abrogated by the excess electrons resulting from the dissociation of protons from the phenolic groups. Scheme I also illustrates the electronic mechanism postulated as responsible for the observed lowering of fixed negative charge density of the epidermal surface at high pH. The groups primarily affected in the upper pH range are likely constituted by the ϵ-amino group of lysine, which together with the guanidyl group of arginine and the phenolic group of tyrosine comprises approximately 13% of the amino acid content of keratin (24). Delocalization of electronic charge originating in the bound hexachlorophene to groups normally dissociating their protons in the upper pH range is postulated to increase their acidic pK's and manifest in a lower net density of fixed charge on the surface. A similar increase in the pK's of titrable groups is apparently responsible for the reported anion-induced



Scheme I—Simplified diagrammatic representation of the postulated electronic inductive mechanisms of hexachlorophene-induced changes in the fixed-charge density of human epidermis

shifts in the position of hydrogen-ion titration curves of wool and soluble proteins in the direction of increased alkalinity along the pH axis (9, 10, 25). The proteins were reported to appear to combine stoichiometrically with both hydrogen ions and the anions inducing the shifts (26, 27).

The possibility of direct electrostatic salt binding between fixed cationic groups on the colloids and hexachlorophene anions cannot be entirely excluded. Such interactions would depend upon the availability of both fixed cationic groups and hexachlorophene anions. The interactions would be expected to manifest in a net reduction of fixed positive charge density and could possibly be contributing to the effects of hexachlorophene observed in the vicinity of pH below 5.7 in which imidazolyl and α-amino groups (9) can be expected to be involved. However, if salt binding of hexachlorophene is contributing to its observed effects, it cannot be resolved from the operation of the inductive mechanisms which have been postulated as primarily responsible.

SUMMARY AND CONCLUSIONS

The results of the present study clearly indicate that the bioelectrometric method is capable of detecting the influence of hexachlorophene on the acid–base binding properties of the colloids constituting the epidermal surface. The application of the method has revealed aspects of the interaction of hexachlorophene, which are undetectable by other more conventional experimental techniques. The mechanistic interpretation of the observed hexachlorophene-induced displacement of the titration curve of the epidermal surface, although speculative, is in general agreement with related results previously reported and the postulations of other investigators.

The further utility of the electrometric method in the investigation of the kinetic and equilibrium interaction behavior of hexachlorophene with human skin *in vivo* and the manner in which the results relate to its substantive antibacterial properties are topics of subsequent reports in the present series.

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