As in the first case we now have six equations in six unknowns—G_B', G_B, G_C, C', s_1, and s_2. While in principle the problem can therefore be solved, one finds that the algebra becomes extremely complicated due to the occurrence of an equation cubic in C'.

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Penicillin G Interactions with Deoxycholic Acid Polymer-Like Structures

By F. ALHAIQUE, C. BOTRÉ, G. LIONETTI, M. MARCHETTI, and F. M. RICCIERI

Conductivity, activity coefficient, and ultraviolet absorption data have shown that an interaction occurs between deoxycholate helical complexes (DCA) and sodium benzylpenicillin (P). The mechanism of this interaction is still unknown, but a merely electrostatic interpretation can be ruled out. Experimental data seem to be consistent with the stereospecific nature of this interaction. The presence of DCA helical complexes increases benzylpenicillin resistance to the hydrolytic activity of penicillinase. No interaction and therefore no decrease in the hydrolytic activity of penicillinase has been found with different derivatives, *i.e.*, dimethoxyphenyl- and α-aminobenzylpenicillin. At the same time dehydrocholic acid sodium salt (NaDHCA), which in no case forms helical complexes, shows no effect upon P, whereas sodium salt of cholic acid (NaCA) still exerts a protective action upon P but to a lesser degree.

THE FORMATION of a helical complex of macromolecular dimension from sodium deoxycholate has been previously described by Rich and Blow (1, 2). Under appropriate conditions sodium deoxycholate aggregates in solution to form a gel which behaves in many ways like a polymer of high molecular weight. The fibers, which can be drawn from the solutions, showed an X-ray diffraction pattern which is characteristic of a helical aggregate (2). In a previous work (3) the influence of cation dimensions, hydration, and hydrogen bonds on the thermal stability of deoxycholic acid (DCA) "polymer-like" structure was discussed. It was shown that the thermal stability of deoxycholate aggregates is cationic species dependent.

In this paper the possibility of an interaction between DCA or related molecules and antibiotics has been studied. Even if the micelle-forming properties of deoxycholate could account for some of the observed phenomena, the formation of huge micelles with a regular or "crystalline" (2) internal structure seems to be responsible for effects which occur only when a specific system of hydrogen bonding is established within the micelle.

Conductivity, activity coefficient, and ultraviolet absorption data gave evidence that a specific interaction takes place between DCA polymer-like structure and benzylpenicillin (P). The mechanism of this interaction is still unknown, but an interpretation of the experimental data on merely electrostatic grounds can be ruled out.

It seemed sound to suppose that this interaction should somehow affect the biological activity of P, namely, its resistance to the penicillinase hydrolytic activity.

An inclusion, already demonstrated in the case of fatty acids (4, 5), could be proposed as an explanation for the observed decrease of penicillinase activity which has been chosen as reference. At the same time it could be suggested that the other molecular species present in the solution during the formation of the complex, could be attached to the outside of the helical steroid core, as are amino acids and peptides (2).

It cannot be overlooked that the study of this kind of interaction can lead to a more detailed knowledge of the mechanism of action of this class of antibiotics at the level of biomembranes.

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EXPERIMENTAL

Materials—Deoxycholic acid (DCA), dehydrocholic acid (DHCA), and cholic acid (CA) Merck were further crystallized from alcohol before use. Different DCA salts were prepared by neutralizing DCA with solutions of different hydroxides. The DCA salt solutions were then lyophilized. The same procedure was used in preparing DHCA and CA salts.

Quaternary nitrogen bases were prepared by exchanging the corresponding iodides through an ionic exchanger, *i.e.*, Ionenaustauscher III Merck. The solution was then titrated with a standard HCl solution. The absence of halogens from these solutions was checked. A penicillin G sodium salt (P) sample, whose purity was determined by a spectrophotometer (Hitachi Perkin-Elmer model 139) according to a method described elsewhere (6), was used. Penicillinase (Baltimore Biological Laboratory) was a sample containing 2×10^6 units/ml.

All reagents used were of analytical grade.

Procedure—The different DCA salts were prepared by dissolving DCA with an amount of the corresponding hydroxide solution slightly less than that required. The excess of acid was then removed by filtration. The solution was then lyophilized and the DCA salt recovered. Conductivity measurements were carried out in a thermostatic bath, regulated within 0.01°. The complete filling and closing of the cell avoided the evaporation of the solution. A WTW bridge (model LF3) was employed for such measurements.

The pH values were controlled by means of a pH meter (Radiometer pH M4). The activity coefficients were determined by potentiometric titration carried out by means of a coupled permselective membrane electrode system extensively described elsewhere (7). The activity of sodium ions was calculated by means of a calibration curve, which allowed conversion of the recorded E.M.F. values into the activities. This was done stepwise by diluting a sodium chloride solution, recording the E.M.F. at each step, and plotting it against the logarithm of Na^+ ion activity. A linear plot was obtained. The a_{Na^+} values of the solution were calculated from the measured E.M.F. by interpolating the calibration curve. This was done assuming that $\gamma_{Na^+} = \gamma_{\pm NaCl}$ in the calibration solution (8). The penicillinase activity on P was evaluated with the manometric method described by Henry and Housewright (9) and modified by Pollock (10). Each time 3 ml. of a diluted penicillinase solution, corresponding to 300,000 units, was added to 0.5 ml. of different solutions of P in 0.0177 *N* $NaHCO_3$ as a buffer. The temperature was 30° ± 0.01° and the reading of CO_2 development was done every 2 min. All determinations were checked against a blank containing penicillinase inactivated with boiling water.

The ultraviolet absorption measurements were performed by means of a Hitachi Perkin-Elmer apparatus (model 139).

RESULTS AND DISCUSSION

In order to prove that a real interaction occurs between DCA and P, three sets of measurements were carried out, *i.e.*, conductivity, activity coefficient, and ultraviolet absorption. In Fig. 1, specific conductivity χ is plotted against the concentration of the different compounds considered.

Plot A refers to a solution of NaDCA, plot B to a solution of tetrabutylammonium deoxycholate (TBADCA), and plot C to a solution of P. The trend of the resulting plots is linear. In order to correlate the prerequisite of a polymer-like structure of DCA, TBADCA has been chosen because this DCA salt has proved to form no helical complexes in the range of temperatures of the experiments (3).

In Fig. 2 specific conductivity χ is plotted against the concentration of DCA salts in the presence of P (molar ratio 1:1). Plot A refers to a solution containing NaDCA + P in a molar ratio 1:1. Plot B refers to a solution containing TBADCA + P in a molar ratio 1:1. In both sets of measurements the temperature was 25° ± 0.01°.

Plot A of Fig. 2 shows a break point. It is to be noted that even if DCA concentration is the same in both plots, the stability of aggregates is cationic species dependent, as already discussed (3). In order to prove that this discontinuity is due to a

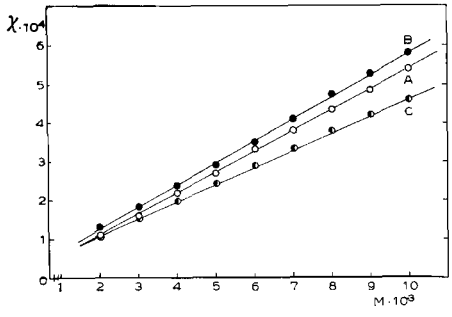

Fig. 1—No discontinuity is detected by conductivity measurements in solutions of different DCA salts. Specific conductivity χ is plotted as a function of the molar concentration of different deoxycholates. Key: A, *a solution of NaDCA;* B, *a solution of TBADCA;* C, *a solution of P.*

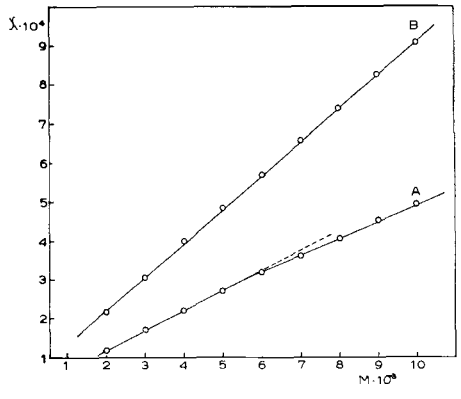

Fig. 2—DCA polymer-like structure interacts with P. No discontinuity is detected when polymer-like structures cannot take place. Specific conductivity χ is plotted against the concentration of DCA salts in the presence of P. Key: A, *NaDCA + P (molar ratio 1:1);* B, *TBADCA + P (molar ratio 1:1). The temperature was 25° ± 0.01°.*

real interaction between P and NaDCA, the activity coefficient of cations in solution was measured through a permselective membrane electrode system described elsewhere (7). The resulting plots are shown in Fig. 3, where plot A refers to a P solution, plot B to a NaDCA solution, and plot C to a NaDCA + P (molar ratio 1:1) solution.

Even in this case the trend of the plot shows a critical point which falls at the same concentration and proves interaction between NaDCA and P.

The increase in cationic activity shown by the complex NaDCA + P would tend to suggest that P interferes with sites of the helical complex, where electrostatic interactions with sodium ions take place. Possibly due to the conformation of the structure there must be a specific nonelectrostatic interaction which results in an increase of cationic activity if compared with that shown by P and DCA, respectively.

Further evidence of a specific interaction was found in extinction coefficient data at a wavelength of 264 mμ. The resulting data are reported in Fig. 4.

As might be expected, the trend of the plots of P and NaDCA taken singularly are linear, whereas

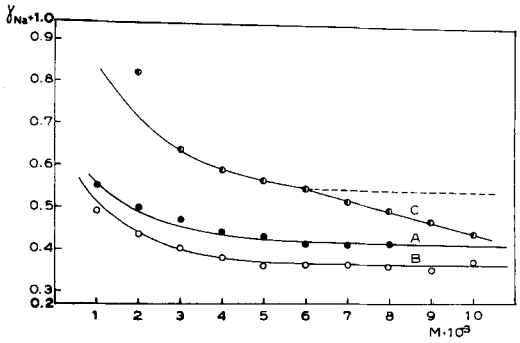

Fig. 3—Activity coefficient measurements show that a real interaction occurs. Activity coefficient γ_{Na^+} is plotted as a function of the molar concentration of a P solution (A), *a NaDCA solution* (B), *and a NaDCA + P solution in a molar ratio 1:1* (C). *The temperature was 25° ± 0.01°.*

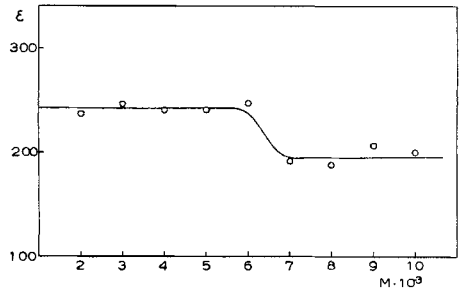

Fig. 4—Ultraviolet absorption data give evidence that DCA polymer-like structure interacts with P at the same value of NaDCA concentration where the transition is observed in both conductivity and activity coefficient measurements. Molar extinction coefficient ε at the wavelength of 264 mμ is plotted as a function of the concentration of a NaDCA + P solution in a molar ratio 1:1. The temperature was 25° ± 0.01°.

the plot referring to NaDCA + P shows a "jump" at the same value of NaDCA concentration where the transition was observed in both conductivity and activity coefficient measurements. The hyperchromic effect is such that the ε value of a diluted solution of NaDCA + P is nearly coincident to that of a penicillin solution when DCA concentration is lower than the transitional value (see Fig. 2). It can then be assumed that the structural changes which occur in deoxycholate complexes are such that no more interaction takes place between NaDCA and P. The same measurements were repeated with different DCA salts. Only the data referring to TBADCA have been reported, but constantly whenever temperature, pH, and ionic strength were such that no helical complex could be established (1–3) no singular points were detected by means of activity coefficient, conductivity, and spectrophotometric measurements. When DCA is replaced with DHCA (dehydrocholic acid), no interaction occurs in a number of concentrations ranging from those of DCA (see Fig. 5). At the same time CA (cholic acid) has the same effects as those for DCA, but the transition occurs at much higher values of concentration. (NaCA concentration was about 2×10^{-2} *M*.) In the attempt to elucidate the mechanism of this interaction, the same kind of measurements were carried out on some derivatives of 6-aminopenicillanic acid. On the assumption that the mechanism would be mainly of a steric nature, a molecule whose structure would ensure a higher degree of rigidity was chosen. In this sense the well known 2,6-dimethoxyphenylpenicillin seemed to be suitable. At the same time in order to find out in which way the total absence of a side chain or the presence of a polar group in it would influence the interaction, the authors took into consideration the α-aminophenylacetyl derivative (ampicillin) and the 6-aminopenicillanic acid itself.

All these compounds show the same behavior, and in the case of ampicillin the resulting data are given in Fig. 6. The plots prove that no interaction takes place. It seems reasonable then to suppose that the interaction between DCA and P is probably of a steric nature.

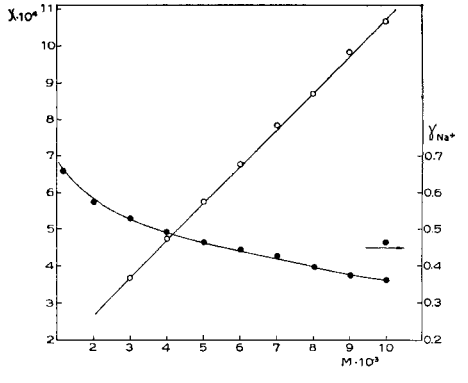

Fig. 5—No interaction occurs in the case of a NaDHCA + P solution. Activity coefficient γ_{Na^+} (●, right ordinate) and specific conductivity χ (○, left ordinate) are plotted against the molar concentration of a NaDHCA + P solution in a molar ratio 1:1. The temperature was 25° ± 0.01°.

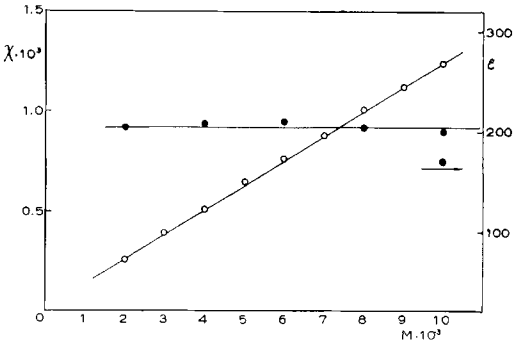

Fig. 6—No singular point can be detected by means of specific conductivity and ultraviolet absorption measurements when benzylpenicillin is replaced with ampicillin. Specific conductivity χ (○, left ordinate) and molar extinction coefficient ε (●, right ordinate) are plotted as a function of the molar concentration of a NaDCA + ampicillin solution in a molar ratio 1:1. The temperature was 25° ± 0.01°.

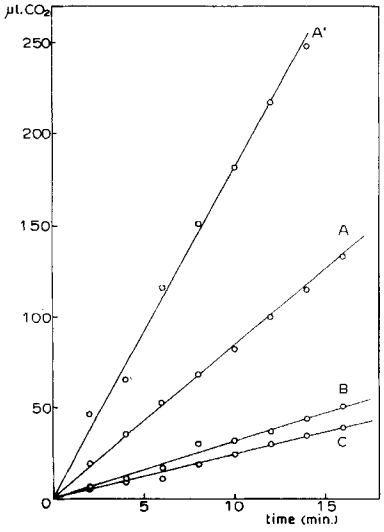

Fig. 7—A protecting action upon benzylpenicillin against hydrolytic activity of penicillinase is shown by DCA polymer-like structures and to a lesser extent by CA. Carbon dioxide volume (μl.) is plotted as a function of time (min.) for a solution of P 1.20 × 10^{-2} M (A), for a solution of NaCA + P 1.20 × 10^{-2} M, respectively (B), for a solution of NaDCA + P 1.20 × 10^{-2} M, respectively (C), and for a solution of NaDHCA + P 1.20 × 10^{-2} M, respectively (A'). The temperature was 30° ± 0.01°. All solutions were buffered with 0.0177 N $NaHCO_3$ (pH = 8.5).

The above described interaction between DCA polymer-like structure and P results in a decrease of penicillinase hydrolytic activity. If the interaction can take place only when DCA has acquired its polymer-like structure, it can be foreseen that there will be a decrease of penicillinase hydrolytic activity only when experimental conditions are such that the formation of a helical complex of macromolecular dimension is possible.

In Fig. 7 the amount of carbon dioxide (μl.) liberated by penicillinase is plotted as a function of time. Plot A refers to a solution of P 1.2 × 10^{-2} *M*. Plot B refers to a solution of NaCA + P 1.2 × 10^{-2} *M*, respectively. Plot C refers to a solution of NaDCA + P both at a concentration of 1.2 × 10^{-2} *M*. In each measurement the temperature was 30° ± 0.01° and the pH = 8.5. The volume of CO_2 developed in the case of NaDCA + P solution is much smaller than for a solution of P. NaCA still exerts a protective action upon P but to a lesser degree. On the other hand, if experimental conditions are such that no interaction can occur, there is no decrease of penicillinase activity as shown in Fig. 8. Here plot C refers to a solution of NaDCA + P both at a concentration of 2.37 × 10^{-3} *M*. Plot B refers to a solution of NaCA + P both at a concentration of 2.37 × 10^{-3} *M* and plot A refers to a solution of P 2.37 × 10^{-3} *M*.

Even if the micelle-forming properties of bile

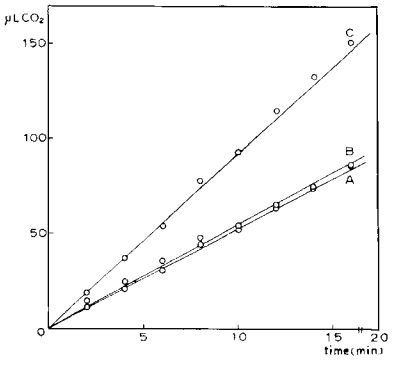

Fig. 8—No protecting action is shown when DCA concentration is lower than the transitional value or when DCA is replaced with CA. Carbon dioxide volume (μl.) is plotted as a function of time (min.) for a solution of P 2.37 × 10^{-3} M (A), for a solution of NaCA + P 2.37 × 10^{-3} M, respectively (B), and for a solution of NaDCA + P 2.37 × 10^{-3} M, respectively (C). The temperature was 30° ± 0.01°. All solutions were buffered with 0.0177 N $NaHCO_3$ (pH = 8.5).

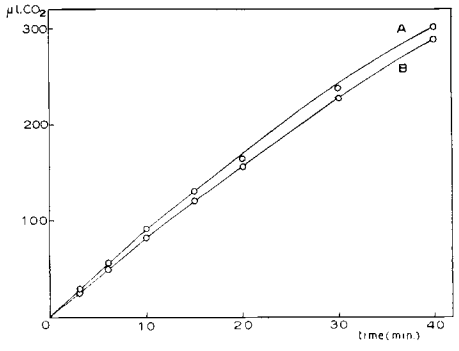

Fig. 9—No variation in penicillinase hydrolytic activity is shown when benzylpenicillin is replaced with ampicillin in a solution of DCA. Carbon dioxide volume (μl.) is plotted as a function of time (min.) for a solution of ampicillin 1.20 × 10^{-2} M (A) and for a solution of ampicillin + NaDCA 1.20 × 10^{-2} M, respectively (B). The temperature was 30° ± 0.01°. All solutions were buffered with 0.0177 N $NaHCO_3$ (pH = 8.5).

salts can account for the observed phenomena (11), nevertheless the ability of deoxycholate to give huge micelles with a regular or crystalline internal structure (2) seems to be responsible for effects which differ in their entity and specificity.

The formation of helical complexes of macromolecular dimension produces a large increase in viscosity which is the outstanding feature of this phenomenon. In the study of changes in viscosity of deoxycholate solutions at different concentrations, Ekwall (12) and Rich and Blow (2) found a number of concentration ranges separated by discontinuities in various properties. These discontinuities are ascribed to changes in the type of micelles. The assumption can be made that the type of DCA structure which corresponds to the range of concentrations indicated by conductivity, UV absorption, and ionic activity measurements interacts with penicillin G. In this respect CA and DCA seem to have almost the same effect upon penicillinase activity (see Fig. 7, plots B and C), but experimental conditions, namely pH = 8.5, suggest that DCA is probably hindered in forming its characteristic complexes which would give a more enhanced effect. In the case of CA, micelle formation and inclusion can be considered as being responsible for the data. This interpretation is consistent with the experimental results which showed that CA interacts with P in a range of concentrations much higher than those of DCA.

At the same time a comparison of the hydrolytic activity of penicillinase upon ampicillin in the presence or absence of DCA helical complexes shows (Fig. 9) that the lack of interaction brings no decrease in the development of CO_2.

In Fig. 8, in the case of a solution of NaDCA + P, where NaDCA concentration is lower than the transitional value, there is an increase in the quantity of CO_2 development if compared with that of a P solution at the same concentration.

We assume that, where no helical complex occurs, the presence of DCA results in an activation of the enzyme, possibly due to Van der Waals interactions with hydrophobic sites of the protein. This effect, of course, is always present, but when helical complexes take place, steric interactions become prevalent. This is further confirmed by the plot A′ of Fig. 7 where NaDHCA, which in no case forms helical complexes (2), shows the same effect upon penicillinase. A solution containing DCA helical complexes can be related on electrostatic grounds to a solution containing chain molecules with high densities of ionized groups.

According to Morawetz (13–15), the electrostatic potentials in the neighborhood of the polyion can be such to induce wide variation in the local concentration of mobile ions. If this is the case, then the presence of DCA helical complexes should have an effect exactly opposite to the one experimentally found on P. Therefore, the polymer-like structure must interact with P molecules through a mechanism which, still unknown, must not be of a merely electrostatic nature.

In conclusion if we choose those experimental conditions defined by that part of the plot of Fig. 2 which is above the break point, we will constantly have a decrease of penicillinase activity and therefore a protective action upon benzylpenicillin.

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