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THE AGGLUTINATION OF ERYTHROCYTES BY CALCIUM PHOSPHATE SOLS

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SUMMARY

The non-specific agglutination by calcium phosphate sols of a range of erythrocytes from various animals has been studied. With all species of erythrocytes chosen, the broad result emerges that the agglutination is controlled by the potential of the sols. The potential can be varied in sign and magnitude by altering the ratio of calcium to phosphate in the precipitating solutions. In particular, agglutination becomes maximal when the sols have their highest positive charge.

Calcium phosphate colloids occur widely in biological systems. The particles are often intimately associated with macromolecules such as collagen. The properties of such sols have been little studied, and it seemed of interest to measure the electrophoretic properties of calcium phosphate sols prepared over a range of calcium/phosphate ratios, and to relate these properties to a simple heterocoagulation process using erythrocytes as a model. This sol/cell system was suggested by earlier unpublished experiments of BANGHAM AND PETHICA on the agglutination of human red cells in saline by colloidal dispersions of uranyl and calcium phosphates, from which it appeared that the surface charge of the colloid was the controlling factor in the agglutination. Non-specific agglutination of erythrocytes has also been little studied, and the experiments described here will be of some value in that attention will be drawn to typical erythrocyte agglutination caused by heterocoagulation with small colloidal particles, a process which is non-specific in comparison with, for example, agglutination by viruses.

EXPERIMENTAL

A Mattson type electrophoresis cell was used with direct illumination. Its calibration has been described by BANGHAM *et al.*[1]. The sols were prepared from analytical grade $CaCl_2$ and Na_2HPO_4. The aqueous solutions of these reagents at 5 mM concentration gave pH values of 5.0 and 7.9 respectively. On mixing, the resulting sols gave pH values in the range 7.1 to 7.9, depending on the ratio of the amounts of the two reagents. These pH values are not recorded individually, since the effect of pH on the electrophoretic mobility of erythrocytes over this range is small,

and because addition of washed red cells tended to buffer the mixtures towards neutral.

The agglutination experiments were carried out with human, bovine, rat (Wistar) and trout (*Salmo trutta fario*) erythrocytes. The cells were collected into citrate or heparin anticoagulant, and washed three times with 145 mM NaCl solution. Agglutination is easily followed by visual observation as with antibodies, and the first experiments to discover convenient experimental conditions were visual. A more quantitative method was then used in which agglutination was followed turbidimetrically with a Unicam SP 600 spectrophotometer. The erythrocytes were added at a final density of approximately 1 vol. of packed cells in 1000 vol. of electrolyte solution to a sol prepared by premixing 10 mM $CaCl_2$ and 10 mM Na_2HPO_4 solutions (both made up in 145 mM NaCl) to the desired ratios. The cells were added immediately after mixing these solutions and the absorbance was followed with time up to 1 h. The readings at 1 h are a convenient end point since the turbidity changes occur principally in the first 5–15 min under the chosen conditions. All data were obtained at 20°.

RESULTS

The experiments on the electrophoresis of calcium phosphate sols were carried out by observing the movements of sol particles of up to about 15 μm in diameter. Particles of this size appear slowly after mixing the reagents in the range of ratios chosen, and 1 h after mixing was found to be a convenient time. Fig. 1 shows the results obtained with these sols in three sets of experiments in which the ratio of calcium to phosphate was varied. In the first, 5 mM solutions of $CaCl_2$ and Na_2HPO_4 were mixed in varying proportions. In the second, the total concentration of $CaCl_2$

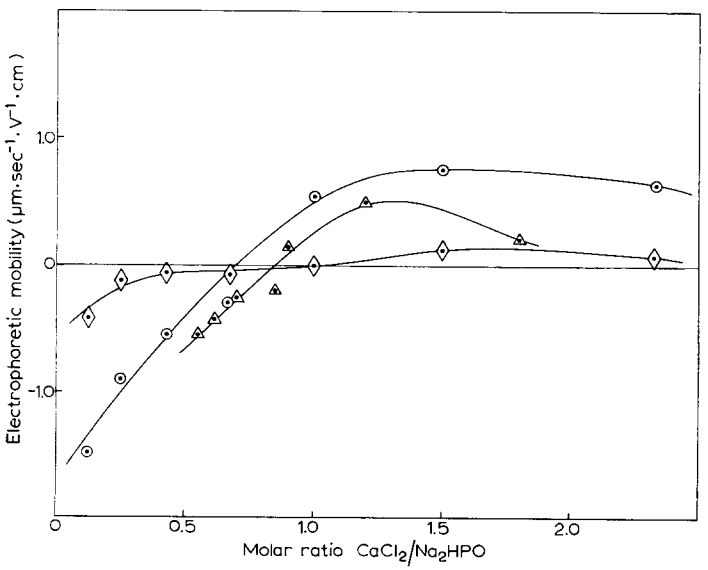

Fig. 1. Electrophoretic mobilities of calcium phosphate sols measured 1 h after mixing solutions of $CaCl_2$ and Na_2HPO_4 at a variety of molar ratios at 20°: ⊙, 5 mM $CaCl_2$ mixed with 5 mM Na_2HPO_4; △, $CaCl_2$ constant at 5 mM, Na_2HPO_4 added to required ratio; ◇, $CaCl_2$ and Na_2HPO_4 both at 10 mM in 145 mM NaCl.

was kept at 5 mM and Na_2HPO_4 was added to the desired ratio. In the third, 10 mM solutions of $CaCl_2$ and Na_2HPO_4, each made up in 145 mM NaCl, were added to each other in varying ratios. The results in Fig. 1 for this third set of experiments are for sols produced as in the agglutination experiments. In all cases, the electrophoretic potentials showed a well defined charge reversal, with negative potentials in the presence of excess phosphate and positive potentials with excess of calcium. The precise electrophoretic potentials will depend on the unknown ratio of free calcium ions to free phosphate ions in the solutions after sol formation. The addition of NaCl, as expected, lowers the potentials, but the reversal of charge is still found. The data of Fig. 1 broadly resemble the electrophoretic pattern for sols such as AgI, in which the potential-determining ions make up the solid phase. On the positive side, there is clear evidence of a maximum in the potentials in the presence of excess calcium. This again parallels features of the well-known AgI sol system.

The electrophoretic mobility of human erythrocytes suspended in 145 mM NaCl was found to be -1.05 $\mu m \cdot sec^{-1} \cdot V^{-1} \cdot cm$, in good agreement with published values[2,3]. Under the same conditions, bovine, rat and trout erythrocytes gave mobilities of -1.04, -0.97 and -0.61 $\mu m \cdot sec^{-1} \cdot V^{-1} \cdot cm$ respectively. Bovine cells suspended in 145 mM NaCl + 10 mM $CaCl_2$ (pH 5.8) gave a mobility of -1.02 as against a value of -1.34 suspended in 145 mM NaCl + 10 mM Na_2HPO_4 (pH 7.8). It follows that the erythrocytes remain negatively charged during the agglutination experiments, so far as the effect of free calcium and phosphate ions are concerned.

The agglutination results are shown in Fig. 2. In all cases, a broad zone of

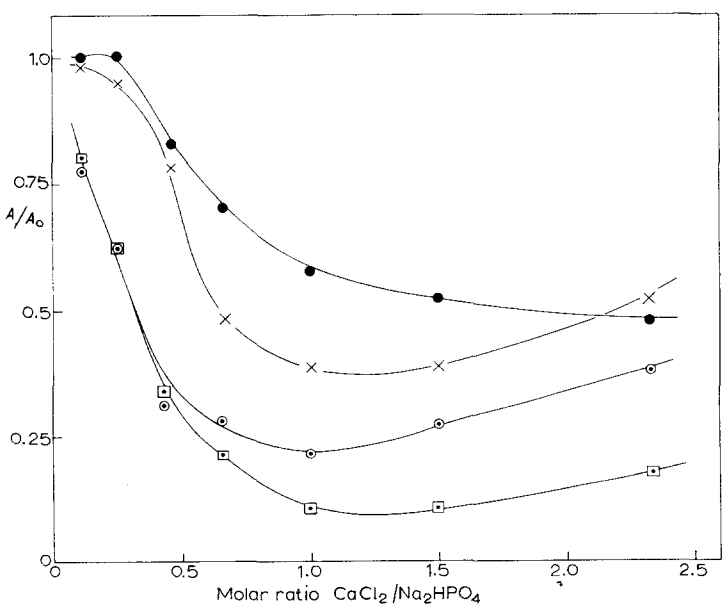

Fig. 2. Agglutination of erythrocyte suspensions by calcium phosphate sols formed at a variety of $CaCl_2/Na_2HPO_4$ ratios at 20°. NaCl concn. 145 mM. Agglutination recorded as a decrease in absorbance 1 h after mixing with sol-forming solutions of $CaCl_2$ and Na_2HPO_4 each 10 mM in 145 mM NaCl, and shown as A/A_0, where A_0 is the initial absorbance and A its value after 1 h. ●, Bovine; ×, rat; ⊡, trout; ⊙ human.

maximum agglutination is observed, corresponding with the zone of positive potentials for the calcium phosphate sols. Electrophoretic observation of clumps of agglutinated cells showed that their overall charge was slightly negative at a 1:1 ratio of calcium to phosphate. This shows that the cells are carrying many colloidal particles, and that the agglutination is caused by particle bridging. It was also frequently observed that cell clumps were attached to the walls of the glass test tubes, demonstrating a further type of heterocoagulation in which negatively charged cells are attached to the negatively charged glass by bridging with positive sol particles. After agglutination, slight slow haemolysis was observed. The effect of haemoglobin on the agglutination was tested with bovine cells by adding bovine haemoglobin to sols formed at 1:1 calcium to phosphate. With increasing amounts of haemoglobin, the agglutination was increasingly inhibited and finally prevented, due to the adsorption of the protein on the sol particles.

The results show that the charge on calcium phosphate sols can be varied in accordance with rules that are well-established in colloid chemistry, and that the interaction of the sol particles with typical biological surfaces such as erythocyte membranes can lead to simple non-specific agglutination in qualitative accord with the same rules. The electrostatic interaction between particles of like sign is always repulsive if the magnitude of the two potentials is similar[4]. When one of the negative potentials is much smaller than the other, attractive electrostatic interactions become possible[5,6]. If the signs of the potentials are opposite, the electrostatic interaction is always attractive. Correspondingly, in the agglutination experiments, the agglutination is pronounced with calcium phosphate sols of low negative potential, and becomes maximal at positive potentials, the red cells themselves retaining a relatively high negative potential throughout. This non-specific electrostatic heterocoagulation resembles, for example, the mutual attachment of erythrocytes and positively charged trypanosomes observed to occur when the trypanosome motility is reduced by cellular degeneration or cooling[7]. The mutual adhesion of cells of similar overall potential is governed by more specific forces[6]. In view of the almost ubiquitous presence of low levels of inorganic phosphate in extra-cellular solutions, agglutination or electrophoretic measurements in the presence of metal ions (*e.g.* Al^{3+}) able to produce phosphate sols are subject to considerable error in experiment and interpretation.

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