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Short Communications

On the Adsorption of Proteins on Calcium Phosphate

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The use of calcium phosphate for purification of proteins is well established. There appears, however, to exist some confusion concerning the effect of pH on the adsorption of proteins on this adsorbents. Thus there are reviews [1,2] on enzyme purification which state that the adsorption always increases with a decrease of pH, whereas others [3] state that maximum adsorption occurs at the isoelectric point of the protein. In support of the latter statement Singer and Kearney [4] found that L-amino acid oxidase from snake venom was maximally adsorbed on calcium phosphate gel at the pH where the crude enzyme had its minimum solubility in ammonium sulfate solutions. However, the solubility minimum of an impure protein may not be identical with its isoelectric point due to protein-protein interaction [5] with impurities. Therefore the effect of pH on the adsorption of pure proteins of known isoelectric points on calcium phosphate gel has now been studied. An effect of certain anions on the adsorption was also noticed.

Preliminary experiments with an acid, neutral and a basic protein (Fig. 1) gave results in agreement with the theory that maximum adsorption occurred at the isoelectric point (IP) of the protein *. Thus metmyoglobin (IP 6.8) was maximally adsorbed around pH 6.8, whereas the acid protein, egg albumin (IP 4.7) showed a decrease in adsorption with increasing pH and the basic protein, cytochrome c (IP 10.7) showed the opposite effect.

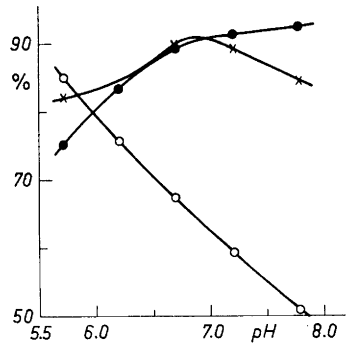

Fig. 1. Effect of pH on the adsorption of proteins on CPG. The test system, of final volume 5 ml, contained 8 mg CPG plus phosphate buffer, $\Gamma/2$ 0.01. The reaction time was 30 min. ○ Egg albumin, 2.0 mg; × Metmyoglobin, 0.66 mg; ● Cytochrome c, 0.30 mg.

Further experiments were carried out with oxyhemoglobin (IP 6.7). It was observed that the adsorption was very rapid (maximum adsorption occurred within 1 min). It was verified that the adsorption of oxyhemoglobin was reversible (the adsorbed protein could be eluted by an alkaline phosphate buffer) and that the adsorption followed Freundlich's adsorption equation[6]. When the effect of ionic strength and pH on the adsorption was studied, (Fig. 2) maximum adsorption was obtained around the isoelectric point of the protein at comparatively low ionic strength (0.01). When the latter was increased by addition of phosphate, the adsorption was diminished, but only at the higher pH values studied. At the

* The isoelectric points cited in this paper refer to electrophoretically determined values (usually at an ionic strength of 0.01). They have been compiled from review articles [7-9].

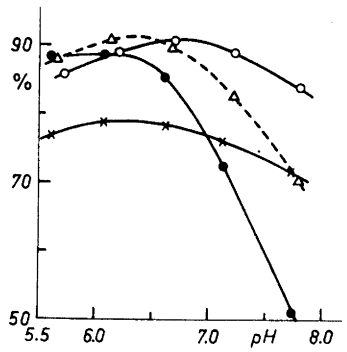

Fig. 2. Adsorption of oxyhemoglobin on CPG. The test system contained 1.67 mg horse oxyhemoglobin and phosphate or sodium chloride as indicated. The reaction time was 5 min. Other conditions as in Fig. 1. ○ phosphate, $\Gamma/2$ 0.01; △ phosphate, $\Gamma/2$ 0.02; ● phosphate, $\Gamma/2$ 0.04; × phosphate, $\Gamma/2$ 0.01 + chloride, $\Gamma/2$ 0.03.

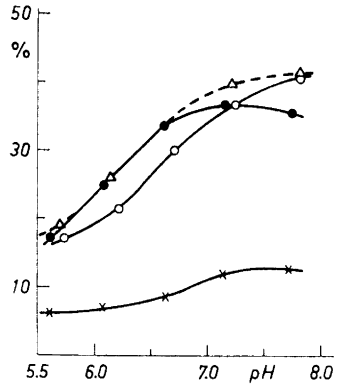

Fig. 3. Adsorption of lysozyme on CPG. The system contained 2.0 mg lysozyme instead of oxyhemoglobin with other conditions as in Fig. 2. Curves indicated as in Fig. 2.

most acid pH a slight increase in adsorption was actually observed. The result was a shift of the point of maximum adsorption to the acid side of the isoelectric point by an increase of phosphate concentration. Chloride, on the other hand, decreased the adsorption over the entire pH range studied (Fig. 2). Fluoride was found to behave as phosphate, whereas sulfate behaved as chloride.

Similar experiments were also carried out with lysozyme (IP 11.0) and bovine serum albumin (IP 4.7). Lysozyme (Fig. 3) behaved similarly to oxyhemoglobin with respect to phosphate and chloride, but serum albumin (Fig. 4) behaved differently. In the latter case, phosphate depressed the adsorption over the entire pH range studied, whereas chloride had a small depressing effect at the lower pH values, which changed to an increase in adsorption at pH 7.8.

Thus, also the results obtained with oxyhemoglobin, lysozyme and bovine serum albumin are in accordance with the theory that maximum adsorption occurs at the isoelectric point of the protein. The different behaviour of the anions studied with respect to their effect on the adsorption of certain proteins is, however, difficult to explain from the data available. Both anion-protein and anion-adsorbent interaction have to be considered. It may be recalled in this connection that phosphate ions combine with oxyhemoglobin [8], making the protein molecule more negatively charged. This could explain why the point of maximum adsorption of this protein is depressed to lower pH-values with increasing phosphate concentration.

Finally, it should be pointed out that advantage may be taken of such anion effects in purification experiments with calcium phosphate.

Material and methods. Calcium phosphate gel (CPG) was prepared according to Tsuboi and

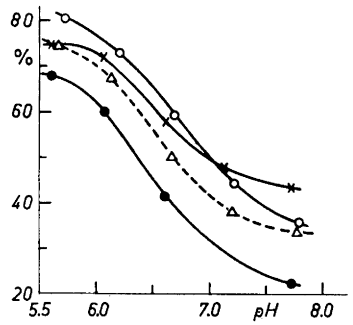

Fig. 4. Adsorption of bovine serum albumin on CPG. Test system contained 2.0 mg bovine serum albumin and only 4 mg CPG. Other conditions and curves indicated as in Fig. 2

Hudson [10], horse oxyhemoglobin was prepared according to Ferry *et al.* [11] and beef cytochrome c according to Neilands [12]. Crystalline beef metmyoglobin was a gift from Mr. Å. Åkeson. Egg albumine, lysozyme and bovine serum albumine were crystalline commercial preparations. Phosphate buffers were prepared from the sodium salts and when other anions were studied the corresponding sodium salts were also used in order to avoid a cation effect on the adsorption phenomena. The adsorption experiments were carried out at + 4°C in 10 ml centrifuge tubes which were agitated on a simple manual rotator during the incubation. The adsorbent was then centrifuged off and protein remaining in the supernatant solution was determined from the adsorption at 280 mμ (or 405 mμ in case of the heme proteins) corrected for a blank, obtained by omitting the protein from the test system. Only a rather narrow pH-range (5.6−7.8) was studied as calcium phosphate is unstable outside this range. All experiments were carried out in the presence of phosphate buffer, as it was found impossible to free the gel from free phosphate by repeated washing on the centrifuge. For an explanation of this effect se Ref. [13,14]

Preliminary experiments were carried out with a reaction time of 30 min, but as it was later observed that the adsorption of proteins on the gel was very rapid, a shorter reaction time (5 min) was used in the following experiments.

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Halogen-Metal Interconversion with Dibromobithienyls

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Bithienyl dicarboxylic acids are useful intermediates for a study of optically active bithienyls [1] and of thiophene analogues to fluorene. Most bithienyls have been prepared by the Ullmann reaction [2-4], which usually gives low yields, especially in the absence of activating substituents such as $-NO_2$ or $-CO_2CH_3$. The coupling of Grignard reagents with $CuCl_2$ was used in a few cases by Steinkopf *et al.*[5,6] for the preparation of bithienyls. In the thiophene series, however, organolithium compounds are available through halogen-metal interconversion, in cases where Grignard reagents are formed only with difficulty [7,8]. Thus the present author and Karlsson [9] were able to demonstrate that good yields of bithienyls can be obtained on coupling 2- and 3-thienyllithium with $CuCl_2$.

It has now been found that 4,4′-dibromo-3,3′-bithienyl (I) and 3,3′-dibromo-2,2′-bithienyl (II) are obtained in 50−60 % yield by treating 4-bromo-3-thienyllithium and 3-bromo-2-thienyllithium, respectively, with $CuCl_2$ at −70°. The NMR-spectra of the products showed that no rearrangement had occurred during the coupling. II was obtained earlier in lower yield by Steinkopf *et al.*[6] by the coupling of 3-bromo-2-thiophenemagnesium bromide, obtained through the entrainment Grignard reaction of 2,3-dibromothiophene, with $CuCl_2$.