Countercurrent Distribution of Phosvitin*

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ABSTRACT: Phosvitin can be fractionated into two components by countercurrent distribution with an aqueous solvent system containing dextran, polyethylene glycol, phosphate buffer, and a high concentration of sodium chloride. After nearly complete separation of the components by over 200 transfers, they may be separated from the polymers of the solvent system by ion-exchange chromatography and from the inorganic salts by gel filtration. Upon redistribution, the two components are characterized by the same partition ratios that govern their original distribution. They have similar contents of nitrogen and phosphorus. At least one of the two phosvitin fractions appears to be homogeneous, as suggested by its nearly ideal distribution.

Chromatography of phosvitin on DEAE-cellulose columns had been shown to result in its fractionation into two components (Connelly and Taborsky, 1961). More recently, we observed that the manner of this fractionation depends on the precise nature of the ion exchanger. This finding (to be discussed below) appeared to restrict the general utility of the chromatographic procedure and we sought a new method for the investigation of the composition of phosvitin preparations. In this report, we describe the resolution of phosvitin into two components by countercurrent distribution, and outline a procedure for the isolation of these phosvitin fractions.

Materials and Methods

Phosvitin was prepared by the methods of Joubert and Cook (1958), or of Mecham and Olcott (1949). It was rendered metal free and was stored as described earlier (Taborsky, 1963). Elementary analyses were done by procedures cited previously (Connelly and Taborsky, 1961) and the analytical data are expressed on a moisture-free basis. Dextran 500 (Pharmacia), in aqueous solution, was treated with sodium borohydride and then precipitated with ethanol; polyethylene glycol (Carbowax 6000, Union Carbide) was dissolved in acetone and then precipitated with ether. Both procedures were described by Albertsson (1960) and were effective in removing impurities that absorb in the ultraviolet region. All other reagents were the best commercially available grades and were used without further purification. Glass-distilled water was used.

The dextran–polyethylene glycol system was described by Albertsson (1960).[1] The solvent system adopted for use in the distribution trains consisted of a given weight of an aqueous mixture, containing 14.1% dextran and 8.8% polyethylene glycol (w/w), added to an equal weight of 0.01 M acetic acid–sodium acetate buffer (pH 4). In this mixture, an amount of NaCl was dissolved to yield an average concentration in both phases of 3.5 M. The ratio of the volume of the upper phase (polyethylene glycol rich) to that of the lower phase (dextran rich) was about 1:2. The effect of pH and salt concentration on the partition of the protein was studied in test tube experiments with appropriately modified solvent systems.

The countercurrent distribution trains (a manually operated 30-tube train and a 200-tube train operated by an automatic robot) were of the type developed by Craig and Post (1949). In the long train, an upper phase "forerun" and a lower phase "cocurrent" (0.5 ml/tube) were employed. The distribution trains were operated in the "fundamental" manner. Since even the fastest protein component moved far behind the front of the upper phase, it was possible to recycle the upper phase for a significant number of additional transfers beyond the basic capacity of the train. Equilibration was accomplished with 45 rocking cycles. With the 30-tube train, the phases were allowed to separate for 15 min with the tubes in a completely horizontal position (in order to maintain maximal solvent interface) followed by 5 min in the customary settling position. With the 200-tube train, the settling period was lengthened to 60 min (the tubes being in the usual settling position throughout this period). Experiments were carried out at

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[1] A large number of organic solvent systems were tested for their potential usefulness in the countercurrent distribution analysis of phosvitin since it was expected that the recovery of protein from an organic solvent system would be a simpler procedure than its separation from other polymeric material in the aqueous system; these tests were unpromising because either the protein was insufficiently soluble or the great bulk of the protein moved with the water-rich phase.

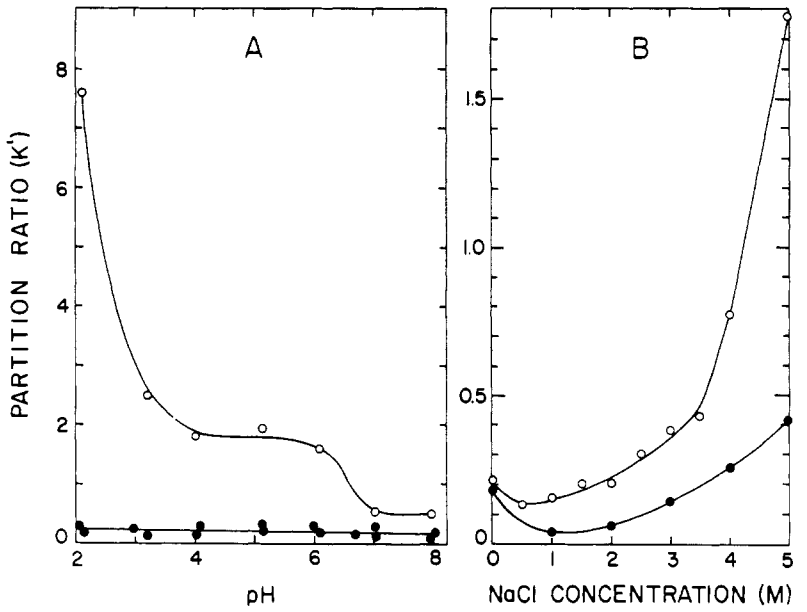

FIGURE 1: Variation of the partition ratio of phosvitin as a function of pH and salt concentration. The buffer solutions were HCl–KCl (pH 2–3), acetic acid–sodium acetate (pH 4), or potassium phosphate (pH 5–8). The total buffer concentration was about 0.01 M in all cases. The pH values shown are those of the buffer solutions measured prior to mixing with the polymer solution. These values are about 0.1–0.3 pH units lower than the corresponding values obtained for the complete solvent phases. A: ●, no added salt; ○, 5 M NaCl. B: ●, pH 7; ○, pH 4. About 15 mg of phosvitin were used in each experiment. For other details see text.

room temperature. In general, all of the experiments and theoretical calculations were closely patterned to the procedures and suggestions of Craig (1960) and King and Craig (1962).

Spectrophotometric analysis of the tube contents was somewhat cumbersome because the solutions were frequently cloudy, even after centrifugation. Cooling or dilution of the samples helped to produce optically clear solutions. Because neither the solvent nor the protein solutions appeared to obey Beer's law strictly, and because of the occasional need for dilution, spectrophotometrically determined partition ratios have only relative significance, within a given experiment.

Recovery of the protein was accomplished by dilution of the mixed-phase system with water to reduce the salt concentration to 0.1 M, followed by adsorption of the protein onto DEAE-cellulose columns (2.5 × 35 cm) prepared similarly to those described earlier (Connelly and Taborsky, 1961) except that 0.01 M sodium Veronal buffer (pH 7) was used instead of Tris-HCl buffer (pH 8). After all of the dilute solution (up to 12 l.) was passed through a given column, it was washed with several column volumes of the buffer. The effluent collected up to this point contained no protein (as judged by the absence of ultraviolet-absorbing material). While the early portion of the effluent that was collected during the passage of the diluted solvent system through the column contained both dextran and polyethylene glycol [as determined qualitatively according to Morris (1948) and Oliver and Preston (1949), respectively], the last part of the effluent obtained during the washing with buffer was free of both of these polymers. The protein was then eluted with buffer that contained 0.5 M NaCl. The protein solution (in about 100 ml of effluent buffer) was, in turn, passed through a Sephadex column (Pharmacia, G-25, medium, column volume about 500 ml, flow rate 1–2 ml/min) and the effluent was analyzed for protein spectrophotometrically and for salt conductometrically. In order to render the protein essentially salt free, the solutions were subjected to gel filtration twice. The protein fractions were combined and lyophilized.

Chromatographic protein fractionation procedures were the same as those described earlier (Connelly and Taborsky, 1961). All chromatographic and gel filtration experiments were done at about 2°.

Results

Variation of the Partition Ratio as a Function of pH, Salt Concentration, and Protein Concentration. Figure 1A shows that the distribution of phosvitin between the two phases of the dextran–polyethylene glycol system is dependent on pH when the salt concentration is high but not when it is low. The pH is nearly the same (average deviation from the mean, 0.02) in the two phases of all of the solvent systems used in this experiment. The pH dependence of the distribution appears to be due primarily to a pH effect as such and not to specific buffer effects since nearly the same values of K' were noted when partition ratios obtained with solvent systems containing different buffers of identical pH were com-

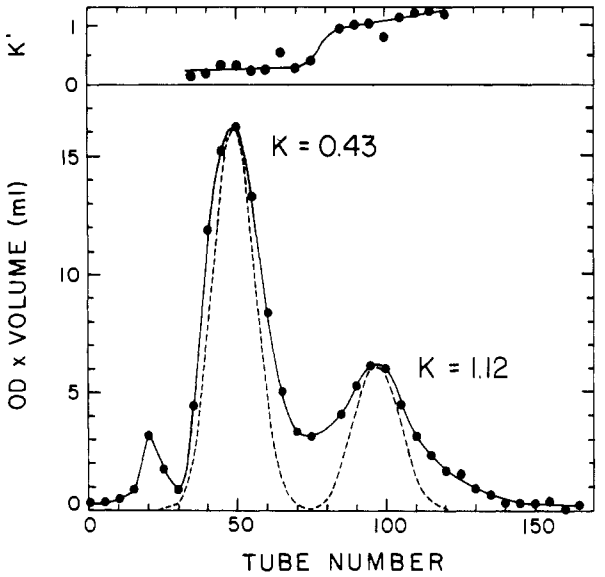

FIGURE 2: Distribution of phosvitin (3 g) by 241 transfers. For experimental details, see text. The partition ratios shown next to the two major "peaks" were calculated for the theoretical distribution pattern, depicted by the broken line. The ordinate shows the sum of the products of the optical density of each phase and of its respective volume, in a given tube: this value is a measure of the total amount of protein in each tube. At the top of the figure, the measured partition ratios are given as a function of tube number.

pared at about pH 3, 4, and 6. Anticipating the result yet to be discussed, that phosvitin can be resolved into two components by countercurrent distribution, it may be noted here that the distribution of one of these components (of low partition ratio; see below) is characterized by a qualitatively similar dependence on pH. That the partition ratio of the protein is highly sensitive to the concentration of salt is shown also in Figure 1B. In contrast, variation of the quantity of protein appears to be without marked effect on the distribution of phosvitin between the solvent phases. In test tube experiments involving two transfers, the partition ratios for tubes 0–2, respectively, were about 0.3, 0.7, and 1.4, whether 27, 40, 61, or 120 mg of protein was partitioned. These partition ratios were nearly unchanged 19 hr later.

Countercurrent Distribution of Phosvitin and Redistribution of the Isolated Phosvitin Components. Figure 2 shows the results of a countercurrent distribution experiment in which phosvitin (a preparation according to Joubert and Cook, 1958) was subjected to distribution with 241 transfers. One minor and two major components (centered about tubes 20, 49, and 97, respectively) were resolved without extensive overlap. Figure 2 shows also the variation of the measured partition ratio as a function of tube number. This ratio is reasonably constant (about 0.3) through the region in which the larger of the two major fractions is found (tubes 35–75). An apparently significant although slight trend upward (from about 1.0 to 1.2) may be noted in the value of the partition ratio through the region containing the smaller of the two major fractions (tubes 85–120). The broken line represents the distribution pattern calculated for components behaving "ideally." The calculated partition ratios of 0.43 and 1.12 are reasonably close to the actually measured values of 0.3 and 1.0–1.2, respectively.

The two major components were isolated and redistributed with 30 transfers each. Redistribution was carried out with only 30 transfers since the demand of a long-train experiment on quantity of material would have been excessive and the short-train experiment appeared to suffice for a demonstration of the reproducibility of the manner of partition of the components. These experiments are described in Figure 3. The distribution of previously unfractionated phosvitin is shown for comparison. The calculated partition ratios (0.48 and 1.11) agree very well with those calculated for the original distribution pattern (0.43 and 1.12).

The ratio of the two major components of phosvitin is nearly 2:1, based on the areas of the respective peaks shown in Figure 2. No attempt was made to obtain maximal yield in isolating these components since it was convenient, in view of the large volumes of solutions involved in the numerous chromatographic steps, to work with only such fractions which contained relatively high concentrations of protein.

In terms of elementary composition, the two major phosvitin components differ only slightly. One of the two ($K' = 0.4$) was found to contain 11.4% N and 10.0% P, while the other ($K' = 1.1$) contained 12.7% N and 8.7% P. These values may be compared with the elementary composition of the unfractionated protein: 11.9% N and 9.2% P. Although the phosvitin used in these experiments was essentially free of Fe, the two components contained about 0.1% Fe each, probably sequestered from the large quantities of salt with which

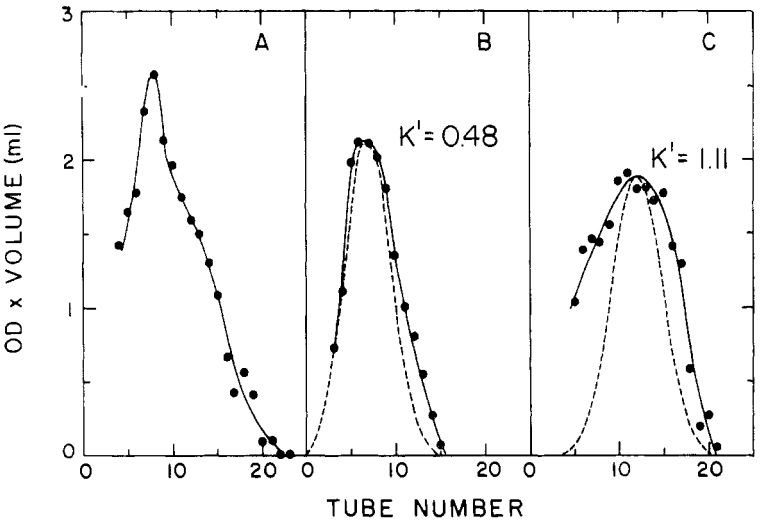

FIGURE 3: Distribution of phosvitin and its components by 30 transfers. For experimental details see text. The partition ratios were calculated for the respective theoretical distribution patterns, shown by the broken lines. A, 84 mg of phosvitin; B, 88 mg of the first major fraction obtained in the experiment described in Figure 2; C, 31 mg of the second major fraction obtained in the experiment described in Figure 2. For the significance of the units along the ordinate, refer to Figure 2. Note that the absolute ordinate values are not comparable between the three experiments for the reasons given in Materials and Methods.

they had been in contact throughout their distribution and isolation.

It is of interest that when phosvitin, prepared according to Mecham and Olcott (1949), was subjected to countercurrent distribution (by 287 transfers), the result was completely analogous to the result obtained with the Joubert and Cook (1958) preparation (the latter being shown in Figure 2). The resolution of material into one minor and two major components and the calculated and measured partition ratios (including the slight upward trend in the value of K' across the band representing the fast-moving major fraction) were all in essential agreement for the two phosvitin samples.

In an experiment similar to the one depicted in Figure 2, the pH and the conductivity of the two phases was also measured upon completion of the distribution. It was found that there was a small but consistent difference between the two phases with respect to both parameters throughout the distribution train. The lower phase was less acidic by about 0.1 pH unit and was characterized by a conductivity about 10% lower than the upper phase, in most of the tubes of the train.

Chromatographic Fractionation of Phosvitin. Mention of our recent chromatographic data which led us to seek a new method of phosvitin fractionation may be of interest, since chromatography of phosvitin on DEAE-cellulose has been used occasionally (*e.g.*, Heald and McLachlan, 1963, 1964; Pinna *et al.*, 1963). All of the original work on the chromatography of phosvitin (Connelly and Taborsky, 1961) was done with columns of DEAE-cellulose A[2] which permitted elution of nearly all of the phosphoprotein in two steps with 0.30 and 0.35 M NaCl (in 0.005 M Tris–HCl, pH 8) when the NaCl concentration in the eluting buffer was increased in increments of 0.05 M, between 0.20 and 0.40 M. Rechromatography of the bulk of the first fraction or all of the second fraction showed that "true" fractionation had been achieved. We have confirmed this manner of fractionation more recently with the same DEAE-cellulose (A), but when columns packed with currently available DEAE-cellulose preparations (B–D)[2] were used, it appeared that the precise manner of elution was largely a function of the type of ion exchanger. The various preparations of DEAE cellulose showed an affinity for phosvitin which decreased in the order A > B > C > D, with increasing amounts of the phosphoprotein appearing in the effluent already in the 0.25 M salt step. For example, about 60% of the total eluted material appeared in this step with C and nearly 90% with D. Furthermore, while chromatography on A resulted in a nearly complete resolution of the "true" components of phosvitin, extensive overlapping of these components was observed with B–D in the eluted fractions. This was ascertained by measurements of the absorbance of the effluent fractions at 250 and 280 mμ. The ratio of these absorbance values was not invariant across the

[2] To avoid cumbersome presentation, we refer in the text by the letters A–D to the various DEAE-cellulose preparations, all of them products of the Brown Co., Berlin, N. H. The full description of these preparations is as follows: A, reagent grade, Lot No. 1038, 1.0 mequiv/g; B, Selectacel, standard, Lot No. 1323, 0.89 mequiv/g; C, Selectacel, Type 20, Lot No. 1212, 0.8 mequiv/g; D, Selectacel, Type 40, Lot No. 1262, 0.91 mequiv/g. The manufacturer informed us that A was an "unusual" lot in that it had been prepared with cellulose used in the preparation of B but by the method employed in the preparation of C. Also according to the manufacturer, "standard methods of preparation" have been set up *after* the preparation of A.

width of any of the eluted bands of the protein, but tended to increase gradually with increasing volume of elution. This finding is consistent with heterogeneity of the bands and the fact that of the two "true" phosvitin components, separable on A, the one eluted with 0.3 M salt is characterized by a lower absorbance ratio (250:280 mμ) than the other component eluted with 0.35 M NaCl.

Countercurrent Distribution of Chromatographic Fractions of Phosvitin. Samples of the two chromatographically distinct components of phosvitin (obtained with columns of DEAE-cellulose A), after they had been rendered metal free, were subjected to countercurrent distribution with the short train. The chromatographic fraction that had been eluted witn 0.30 M salt was distributed largely as an apparently single component with a partition ratio of about 0.4. Distribution of the chromatographic fraction that had been eluted with 0.35 M salt appeared to be heterogeneous, the major portion of the material being present in tubes corresponding to a partition ratio of about 1.

Discussion

Phosvitin as usually prepared is known to be heterogeneous by several criteria. Upon free-boundary electrophoresis, an asymmetrical moving boundary (Joubert and Cook, 1958) or even resolution of phosvitin components (Mecham and Olcott, 1949; Sugano, 1957) can be seen. Paper electrophoresis also reveals heterogeneity (McCully *et al.*, 1959; Sundararajan *et al.*, 1960). Ion-exchange chromatography has been used as a preparative method for the fractionation of this phosphoprotein (Connelly and Taborsky, 1961; Pinna *et al.*, 1963; Belitz, 1963; Heald and McLachlan, 1963, 1964). Of these techniques, the chromatographic one would provide the most convenient fractionating procedure except that its utility now appears to be limited by the subtle dependence of the manner of fractionation upon the precise nature of the ion-exchanger preparation and by the poor resolution of the phosvitin components afforded by currently available DEAE-cellulose products. It seems that the adsorption isotherms of the components of phosvitin with respect to these DEAE-cellulose preparations are much more similar than would be desirable for the efficient resolution achieved with an early ion-exchanger product. The method appears to retain a degree of usefulness when only the gross separation of phosvitin from other materials is sought, *e.g.*, in the isolation of the phosphoprotein from serum (Heald and McLachlan, 1963), or in its recovery from the solvent system employed in this study.

The appearance of two major components upon countercurrent distribution of phosvitin and the reproducibility of the partition ratios of the two components upon their isolation and redistribution is consistent with a "true" fractionation of an originally heterogeneous protein. The distribution of the major component ($K' = 0.4$) is close to "ideal" and suggests that it may be a single protein entity. In view of the sluggish establishment of equilibrium with the solvent system used in this work, and in view of the general phenomenon that polymeric substances are not likely to be ideal solutes, deviations from ideal distribution may not be inconsistent with homogeneity. This component ($K' = 0.4$) corresponds to the chromatographic component eluted with 0.30 M salt from columns of DEAE-cellulose A.

A deviation from ideality is more marked with the second major phosvitin component ($K' = 1.1$) for which an asymmetry was observed (Figure 3C). This may reflect some contamination with the other component or some concentration dependence of the partition, too small to detect in a two-transfer test tube experiment. Alternatively, and perhaps most likely, it may be an artifact due to the difficulty, mentioned earlier, of obtaining optically clear solutions, especially in the first several tubes of the countercurrent train. This component ($K' = 1.1$) corresponds to the bulk of the chromatographic component eluted with 0.35 M salt from columns of DEAE-cellulose A.

The somewhat asymmetrical redistribution of the component of high K' (Figure 3C) is unlikely to be a reflection of some transformation, occurring during distribution, by which one apparent component (high K') would be converted into another (low K'). The position of most of the band with high K' corresponds essentially to the position of the leading shoulder only and not to the peak of the pattern yielded by the unfractionated material (Figure 3A). A transformation should have yielded essentially identical patterns in these two experiments. Furthermore, in the case of a transformation, the distribution would be expected to vary with the duration of the experiment. We have found, however, that the over-all partition ratio of phosvitin is essentially invariant over a period of 19 hr, and that an experiment of more than 10-days' duration (Figure 2) does not show more marked asymmetry than does the experiment of only 1-day's duration (Figure 3C). More marked asymmetry, even the disappearance, of the band corresponding to a high K' would be the expected consequence of a transformation in the experiment lasting for 10 days when compared with the 1-day experiment.

The possibility that some interaction between a single protein and a component of the solvent might be responsible for the appearance of more than one band (*cf.* Cann and Goad, 1965) may be excluded on the grounds that, in that case, the isolation of the apparent components and their subsequent redistribution (after reequilibration with respect to pH and ionic strength) would result in patterns indistinguishable from the pattern obtained with the unfractionated material. Figure 3 shows, however, that this is not the case.

A transformation may have occurred, of course, at some stage in the "history" of the protein. In this regard, we can say only that nearly identical distribution patterns have been obtained with two phosvitin preparations which differed in age by 3 years.

It is of interest to note that preparations of phosvitin, according to the methods of Joubert and Cook (1958) and Mecham and Olcott (1949), appear to be indis-

tinguishable by countercurrent distribution.

The pH dependence of the over-all partition ratio of phosvitin is of interest (*cf.* Figure 1). It seems reasonable to assume that the phosphate groups of phosvitin are the major determinants of the acid–base properties of this protein and that these groups have closely similar pK_a values. As a first approximation, therefore, one may view the pH dependence, or the lack of it, of the over-all partition ratio in terms of the model of a simple acid (see Appendix). The pH dependence of K' (over-all), in the presence of salt, appears to reflect the state of ionization of the phosphate groups, the changes in K' occurring in regions of pH where the secondary and primary ionizations of phosphate groups are expected to occur. Qualitatively similar variations of K' with pH have been observed with unfractionated protein and one of its components (low K'). The other component was not tested. In the absence of salt, K' is invariant with pH. When salt is present, an unequal distribution of the salt between the phases occurs (*cf.* Albertsson, 1960; our own finding shows that the conductivity of the two phases differs), possibly causing a differential suppression of the electrostatic effect and, therefore, a difference between the apparent pK_a values of the acid in the two phases. In the presence of salt, we have observed also a small but significant difference between the pH of the two phases. Either difference alone would suffice to cause K' to be pH dependent by definition and the value of K' would vary as a function of the state of ionization of the acid group. In the absence of salt, the pK_a values of the acid may be more nearly the same in the two phases and since the pH of the two phases is also the same (as we have found it to be the case), K' will not vary with pH.

Appendix

Considering the general case of an acid (AH) and assuming that the following equilibria determine the over-all partition of the acid between two solvent phases (the subscripts u and l denoting upper and lower phases, respectively), one may readily obtain several

$$AH_u \rightleftharpoons A_u^- + H_u^+ \quad (1)$$

$$AH_l \rightleftharpoons A_l^- + H_l^+ \quad (2)$$

$$AH_l \rightleftharpoons AH_u \quad (3)$$

$$A_l^- \rightleftharpoons A_u^- \quad (4)$$

$$H_l^+ \rightleftharpoons H_u^+ \quad (5)$$

expressions for the over-all partition ratio (K') in terms of the equilibrium constants characterizing reactions 1–5. For example,

$$K' \equiv \frac{(AH)_u + (A^-)_u}{(AH)_l + (A^-)_l} \quad (6)$$

$$K' = K_{p,AH}\frac{(H^+)_l[(H^+)_u + K_{a,u}]}{(H^+)_u[(H^+)_l + K_{a,l}]} \quad (7)$$

$$K' = K_{p,A^-}\frac{K_{a,l}[(H^+)_u + K_{a,u}]}{K_{a,u}[(H^+)_l + K_{a,l}]} \quad (8)$$

$$K' = \frac{(H^+)_l K_{p,AH} + K_{a,l}K_{p,A^-}}{(H^+)_l + K_{a,l}} \quad (9)$$

$$K' = \frac{K_{p,AH}K_{p,A^-}[(H^+)_u + K_{a,u}]}{(H^+)_u K_{p,A^-} + K_{a,u}K_{p,AH}} \quad (10)$$

where the subscripts a and p refer to the respective acid dissociation reactions and partition processes. These expressions readily yield one relating all of the equilibrium constants to each other

$$\frac{K_{a,l}}{K_{a,u}} = \frac{K_{p,AH}(H^+)_l}{K_{p,A^-}(H^+)_u} \quad (11)$$

where $(H^+)_l/(H^+)_u = 1/K_{p,H^+}$. Equation 11 may be used to describe several limiting cases. If the pK_a of the acid is the same in both phases then the partition ratios of AH and A^- ($K_{p,AH}$ and K_{p,A^-}) will differ only if the pH of the phases is not equal ($K_{p,H^+} \neq 1$). Conversely, if the pH values are the same in the two phases, then the partition ratios of the two species will be unequal only if the pK_a values are different in upper and lower phase, respectively. If members of any two pairs of parameters in eq 11 are equal [$K_{a,l}$ and $K_{a,u}$; $K_{p,AH}$ and K_{p,A^-}; $(H^+)_l$ and $(H^+)_u$] then members of the third pair are also equal according to eq 11. A consequence of this last situation will be that the over-all partition ratio, K' (6–10), will be equal to the partition ratio of either of the two acid species ($K_{p,AH}$ and K_{p,A^-}) and K' will be independent of pH. Otherwise, the plot of K' *vs.* pH will describe an S-shaped curve the midpoint of which will be related to $K_{p,AH}$, K_{p,A^-}, $K_{a,u}$, and $K_{a,l}$ in a simple manner.

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Spectrographic and Chromatographic Resolution of Metalloproteins in Human Serum*

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ABSTRACT: Fractions of dialyzed human serum obtained by gradient chromatography on DEAE-cellulose columns were analyzed for metals by spark emission and atomic absorption spectroscopy. Such measurements required the prior development of a noninterfering eluting buffer and elimination of contaminating metals from the chromatographic system.

Iron, zinc, manganese, and nickel were found reproducibly in specific fractions, presumably as constituents of metalloproteins. Calcium, magnesium, and strontium were also localized, but their association with proteins seems more tenuous, since they are more loosely bound. Zinc is found in three distinct fractions from one of which a protein containing a stoichiometrically significant amount of zinc was partially purified. All three zinc fractions were separated from the activities of serum enzymes previously known to contain zinc.

Most metals are present in serum in such minute concentrations that, until recently, available analytical techniques were not adequate for their study. Furthermore, methods of protein separation used prior to the last decade involved conditions disruptive to protein–metal interactions. It has long been apparent, however, that metals present in blood serum function in conjunction with proteins, and whenever it has been possible to examine this association as, *e.g.*, for siderophilin and ceruloplasmin, areas of major biochemical importance have been uncovered (Putnam, 1960).

With advances in spark, flame emission, and atomic absorption spectroscopy (Vallee, 1960) and with the development of gentle protein separation methods of high resolution such as ion-exchange and molecular sieve chromatography (see review by Sober *et al.*, 1965) these problems have been overcome in principle. However, major technical obstacles remained before these methods could be combined to yield meaningful data.

In the present study, general procedures were developed which permit emission and atomic absorption spectroscopy of proteins obtained by means of chromatographic and electrophoretic protein fractionation methods. This required elimination of significant contamination and development of noninterfering buffer systems.

These general procedures were then applied to the study of dialyzed human serum: first, to determine the distribution of metals in fractions obtained by ion-exchange chromatography on DEAE-cellulose by spark emission spectrography; second, to study in greater detail the distribution of zinc and magnesium among such fractions utilizing the sensitivity of atomic absorption analysis; and finally, to achieve a partial purification of one of the three serum zinc proteins thus detected. Preliminary reports of this work have been given (Himmelhoch *et al.*, 1964).

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