STUDIORUM PROGRESSUS

Fractionation of Proteins with Different Isoelectric Points by Rivanol

Studies on the interaction between serum proteins and acridine dyes[1] and the finding that tobacco mosaic virus can be partly purified by precipitation with rivanol (2-ethoxy-6, 9-diamineacridine lactate[2]) lead to the application of this reagent for fractionation of serum proteins[3–12]. Except for the precipitation of mucopolysaccharide sulfates[13] and the pertussis antigen[14], acridine dyes were used in combination with other reagents only for the fractionation of serum proteins. No attempt was made to achieve a separation of proteins based on the proper adjustment of pH (or ionic strength) of the solvent during precipitation by rivanol. This communication shows the possibility and the conditions for separating proteins with different isoelectric points by precipitation with rivanol.

Methods[15]. The following proteins were used to study the relationship between their isoelectric points and their precipitability by rivanol: trypsin (1 × crystallized), soybean trypsin inhibitor (3 × crystallized), ribonuclease (crystallized) and muramidase (Worthington Biochemical Corp., Freehold, N.J.); papain, α-chymotrypsin and deoxyribonuclease I (Calbiochem, Los Angeles, California); conalbumin and β-lactoglobulin (bovine, 3 × crystallized) (Nutritional Biochemicals, Cleveland, Ohio); ovalbumin (5 × crystallized), bovine serum albumin (crystallized) and γ-globulin (Cohn's fraction II) (Pentex, Inc., Kankakee, Illinois).

0.1 ml of 1% solutions of each protein in 0.01 *M* NaCl was mixed with 3 ml of buffer solutions (pH 4.0–11.5, prepared according to BRITTON and ROBINSON[16] and diluted 3 times with distilled water) and with 0.1 ml of a 0.4% solution of rivanol (Calbiochem). The portion of protein which was precipitated was determined either by measuring the turbidity at 600 nm or by spectrophotometric determination of protein[17] left in solution after removal of the precipitate by centrifugation. The results obtained by either method were identical. When the spectrophotometric method was used, rivanol was first precipitated from the solutions by addition of KBr to a final 0.25 *M* concentration.

For studies concerning the separation of enzymes, the following raw materials were used: defatted cow milk, pancreas powder (Nutritional Biochemicals) and human serum. The pancreas powder (15 g) was extracted with 100 ml of 0.025 *M* NaCl 4 h at 4 °C and the extract was clarified by centrifugation at 8000 rpm for 1 h. The pH of the protein mixtures was adjusted to the lowest values shown on the Figures 4–6 by addition of 1 *M* acetate buffer pH 4.7. The precipitate, formed after 2.5–3.2 volumes of 0.4% rivanol were added to 1 volume of each preparation, was removed by centrifugation and subsequently extracted with 0.25 *M* KBr in order to solubilize proteins while leaving rivanol in the precipitate. The pH of the supernatant fluid after the first precipitation with rivanol was adjusted to the next higher value by addition of 1 *N* NaOH. Thus a gradual precipitation of proteins could be achieved by increasing stepwise the pH of the solutions.

Xanthine oxidase was determined by measuring the rate of formation of urate from hypoxanthine[18].

Lactoperoxidase was assayed in the following way: 2.15 ml of 0.07% benzidine in 0.057% sodium acetate were mixed at 22 °C with 1 ml of the enzyme preparation and with 0.05 ml of 15% H_2O_2. The O.D. at 660 nm was measured 10 min later and it was compared with the O.D. of blank solutions containing either no substrate or no enzyme.

Elastase was determined according to SACHAR et al.[19]. Elastinorcein (Worthington Biochemicals) was used as substrate. The latter was incubated with the enzyme preparations at 37 °C for 6 h. The amount of orcein released was determined spectrophotometrically at 590 nm.

Trypsin was determined in the following way: 0.5 ml of fractions obtained after precipitation with rivanol from the pancreas extract were mixed with 0.4 ml of 0.1 *M* phosphate buffer pH 7.2 and with 2 ml of the hemoglobin substrate solution[20]. The mixtures were incubated at 37 °C for 15 min. Then 1 ml of 50% trichloroacetic acid was added, the precipitate was removed by centrifugation and the O.D. of the supernatant fluid was measured at

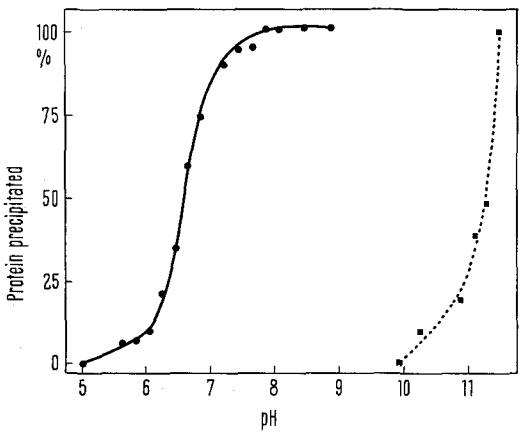

Fig. 1. The pH dependence of precipitation of DNAase I (●) and of RNAase (■) by rivanol. At pH values higher than 11.4 rivanol itself was precipitated.

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[15] Abbreviations used: IP, isoelectric point; IOP, isoionic point; O.D., optical density.
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540 nm. In order to account for any non-trypsin proteolytic activity possibly present in the fractions, the same procedure was repeated, but 3 mg of soybean trypsin inhibitor was added to the samples and the results of the corresponding O.D. measurements were subtracted from the results obtained at the absence of the inhibitor.

Ribonuclease and deoxyribonuclease were determined essentially according to the methods of KALNITSKY et al.[21] and ALLFREY and MIRSKY[22], respectively. Amylase activity was assayed according to the method of BERNFELD[23]. Cholinesterase and muramidase were measured by the methods of RAPPAPORT et al.[24] and SHUGAR[25], respectively.

Results and discussion. The precipitation of proteins by rivanol in relation to their isoelectric points. The precipitation of different proteins by rivanol depends on the pH of the solution. When the portion of precipitated protein was plotted against pH, sigmoid curves were obtained as exemplified by the results shown on Figure 1 for deoxyribonuclease and ribonuclease. When the pH values corresponding to the inflection points of the curves were plotted against the isoelectric points of each protein, results summarized in Figure 2 were obtained. They show that the precipitability of proteins by rivanol is closely related to their isoelectric point and they suggest the possibility of separating proteins, differing in their net electrical charge, by precipitation with this acridine dye.

The occurrence of protein-protein complexes in protein mixtures may influence the course of precipitation. An example of this kind is demonstrated in Figure 3 showing that the precipitation curve of soybean trypsin inhibitor is shifted to higher pH values by the presence of trypsin which itself is not precipitated by rivanol throughout the entire pH range studied.

The possibility of separating proteins by precipitation with rivanol was examined by attempts to fractionate enzymes from suitable raw materials. As shown for lactoperoxidase and xanthine oxidase from milk (Figure 4); deoxyribonuclease, ribonuclease, trypsin and elastase

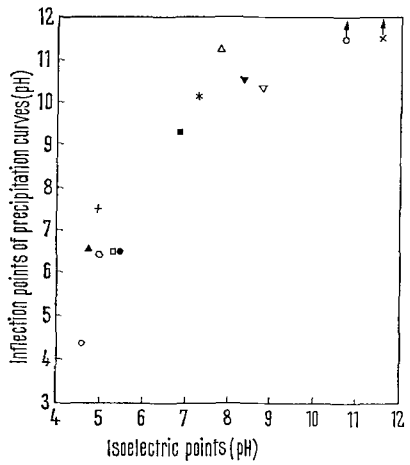

Fig. 2. Relationship between the precipitability of proteins by rivanol and their isoelectric points. The data concerning IP were obtained from references given. Trypsin, ● (not precipitable by rivanol)[26]; α-chymotrypsin, ▼[26]; soybean trypsin inhibitor, ○[27]; DNAase I, ▲[28]; ovalbumin, ◇[29]; muramidase, × (not precipitable by rivanol)[29]; β-lactoglobulin, □[30]; conalbumin, ■[29]; bovine serum albumin ●[30]; bovine γ-globulin, *[30]; trypsin-soybean trypsin inhibitor complex, +[27]; papain, ▽[31]; RNAase, △[32]. At pH higher than 11.4 rivanol itself was precipitated.

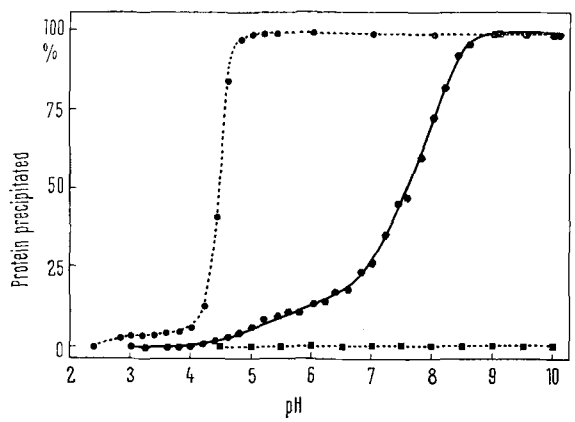

Fig. 3. The pH dependence of precipitation of trypsin (■); soybean trypsin inhibitor (●) and the trypsin-inhibitor complex (●). The preparation of the enzyme-inhibitor complex was obtained by mixing equal parts of 1% solutions of trypsin and of the inhibitor.

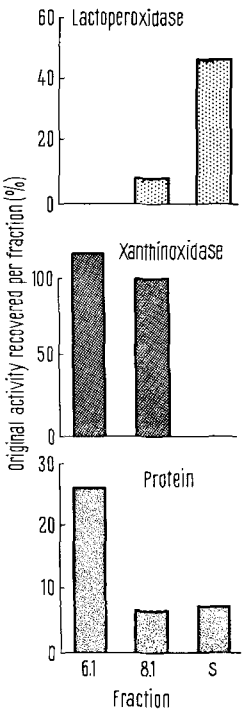

Fig. 4. Distribution of protein, lactoperoxidase (IOP = 9.6)[33] and xanthine oxidase (IP = 5.3–5.4; [34] in fractions obtained after precipitation of milk proteins by rivanol. Designation of fractions: 6.1 and 8.1 = precipitates obtained at pH 6.1 and 8.1, respectively; s = supernatant fluid after precipitation at the highest pH value used in the experiment (8.1).

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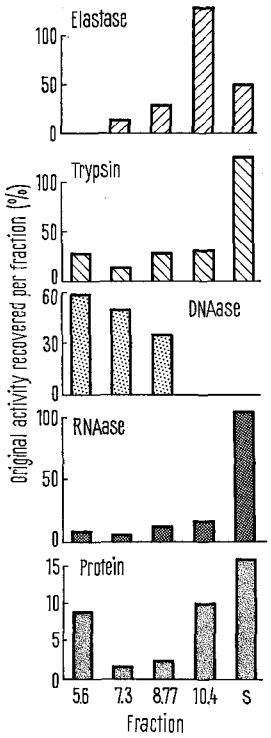

Fig. 5. Distribution of protein, RNAase (IP = 7.8; [32]), DNAase (IP = 4.7; [28]), trypsin (IP = 10.5–11.0; [26]) and elastase (IP = 9.5; [35]) in fractions obtained after precipitation of proteins extracted from bovine pancreas, by rivanol. Concerning the designation of fractions see Figure 4.

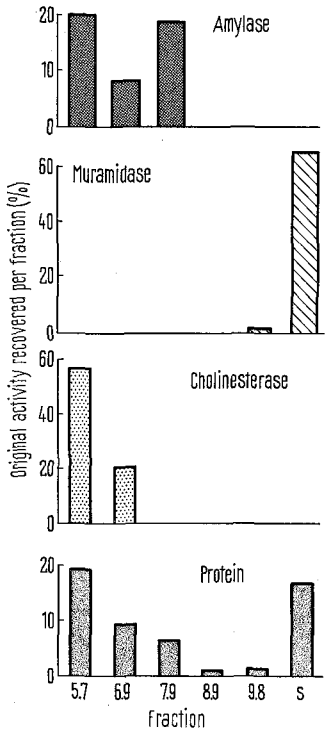

Fig. 6. Distribution of protein, cholinesterase activity, muramidase (IP > 11.0; [29]) and amylase activity in fractions obtained after precipitation of serum proteins by rivanol. Concerning the designation of fractions see Figure 4.

from pancreas (Figure 5) and cholinesterase, amylase and muramidase from serum (Figure 6), partial separation of these enzymes could be achieved. The distribution of enzymes in the different fractions after precipitation was in agreement with their isoelectric points and the relationship shown in Figure 2. The higher than 100% recoveries observed with xanthine oxidase, nucleases, trypsin and elastase can be attributed to the removal of inhibitors, present in crude extracts[26,35,37–39], during the fractionation procedure. Recoveries lower than 100% (Figures 4 and 6) could be ascribed to incomplete solubilization of the enzymes from rivanol-protein precipitates.

In additional experiments it was possible to purify partially muramidase from egg white, tyrosinase from mushrooms and β-amylase from sweet potatoes[36]. Fractionation of proteins could be achieved also by varying the ionic strength of the solvent at constant pH[36].

Interaction between nucleoproteins and rivanol. Histones due to their high isoelectric point are not precipitated by rivanol. On the other hand, nucleic acids are precipitated at low pH values (at pH 2.4 complete precipitation was observed[36]) at which proteins are not precipitated. Therefore, it seemed to be of interest to determine how nucleic acid-histone complexes would behave in the presence of rivanol. The results shown in Figure 7 indicate that rivanol is capable of displacing histones from such complexes. This could be confirmed by the successful extraction with rivanol of histones from calf thymus deoxyribonucleohistone.

The results presented here show that precipitation by rivanol may serve as a useful and fast step in a sequence of procedures designed for the separation and purification of proteins. The purification of bacteriophage T4 muramidase using rivanol, described after this work has been completed[40], serves as an additional example in support of the above conclusions.

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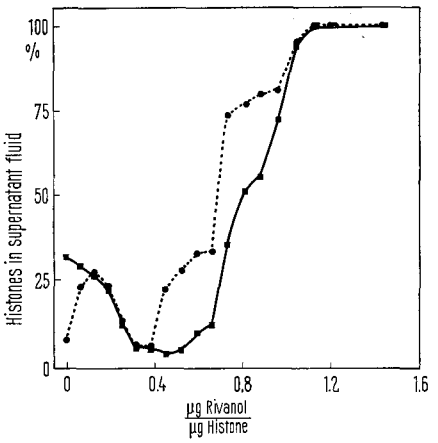

Fig. 7. Competition between rivanol and histones in the reaction with RNA (■) and DNA (●). Equal volumes of nucleic acid (highly polymerized RNA from yeast or calf thymus DNA, 500 µg/ml, both from Calbiochem) and histone (from calf thymus, 250 µg/ml) solutions were mixed and increasing amounts (0–600 µg/ml) of rivanol in 0.03 *M* acetate buffer pH 4.7 were added. The precipitate which formed was removed by centrifugation, rivanol was subsequently removed from the supernatant fluids by precipitation with KBr and the concentration of histones was determined spectrophotometrically[17].

Zusammenfassung. Die Anwendbarkeit von Rivanol als Protein-Fraktionierungsmittel wurde geprüft und an den folgenden Beispielen bewiesen: 1. durch gemeinsame Trennung und teilweise Reinigung folgender Enzyme aus geeigneten Rohmaterialien: Peroxidase und Xanthinoxidase; Desoxyribonuklease I, Ribonuklease, Trypsin und Elastase; Amylase, Cholinesterase und Muramidase; 2. durch Extraktion von Histonen aus Nukleoproteinen.

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PRO EXPERIMENTIS

A Very Simple Trick to Produce Controlled CO_2 Concentrations in the Gas Phase Overlying Cell Cultures

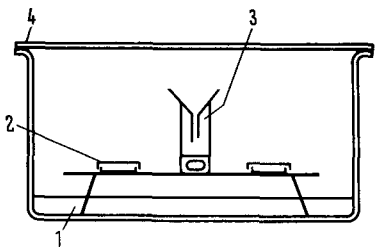
There is more and more need for precise pH control of mammalian cell culture meida. This is usually done by adding carbon dioxide to the air overlying the culture dishes. As experienced by many culturists and emphasized again recently by FERENCZ and NARDONE[1], this requires gas flow incubators and a high rate of gas consumption, which is costly. In addition, these systems do not allow easy adjustments of concentrations of the gas mixture according to needs.

In this laboratory, we are using with success a very simple trick, commonly used by chemists, that obviates all this. In large glass vessels that can be made air-tight, the required amount of CO_2 is produced *after* closing the lid.

In practice, we use large dessicators or aquaria with ground edges and lids. Water is put on the bottom to maintain water vapor saturation. On a tray above the water are placed first the Petri dish cultures, then a narrow high beaker containing normal phosphoric acid. The required amount of sodium bicarbonate (p.a.) is weighed and put in an old-fashioned pharmaceutical *cachet* envelope; just before closing the lid, the cachet is dropped in the acid and a little funnel added to prevent projections. About 1 min later, the cachet envelope is partly dissolved and bubbles of CO_2 appear. Equilibrium is reached within less than $^1/_2$ h and maintained for days.

The CO_2 appears near the ceiling of the sealed chamber; it mixes well with the air and no difference in pH of the medium (as checked by the indicator color) is observed in replicate dishes placed at different levels (up to 10 cm). There is only a very temporary rise of pressure (0.02 Atmosphere in several tests), as the vessels, very tight when the pressure gradient is from outside to inside, allow microbubbles of the gas mixture to escape between lid and vessel even for small pressure differences when the gradient is reversed.

The amount of bicarbonate can be determined from the volume of the vessel. Theoretically a partial pressure of 0.01 Atmosphere at 37 °C will be obtained in a volume



Schematic view of one of the possible arrangements. 1, water; 2, Petri dishes containing the cultures on a tray; 3, vessel containing phosphoric acid and bicarbonate *cachet* with funnel to prevent projections; 4, ground glass lid and edge.

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