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Phosphoproteins from calf-thymus nuclei: Studies on the method of isolation

The studies of LANGAN[1] and of KLEINSMITH and coworkers[2–5] have indicated the possibility that nuclear phosphoproteins play an important role in the regulation of nuclear metabolism. Although general descriptions of the methods utilized in the purification of nuclear phosphoproteins have been published[1,4], no detailed procedure or analysis of the variables involved has been presented. Because of the growing interest in these proteins, the present communication will describe in detail the procedure for isolating the nonhistone phosphoproteins from calf-thymus nuclei. Included in this description will be an examination of the critical variables, some suggestions for improvements of the original methods, and some general comments about the handling of these proteins.

All steps of the purification procedure must be carried out at 4°. It is convenient to start with 8 g dry wt. of nuclei prepared in isotonic sucrose[6], and all volumes of solutions in the following description will be appropriate for this size preparation. However, in cases where a large quantity of phosphoprotein is required, we have found the procedure to be feasible with as much as 60 g of purified nuclei. Each 8 g of nuclei are washed by gently suspending in 1000 ml of 0.01 M Tris (pH 7.5), 3 mM $MgCl_2$ using a motor-driven stirrer at 1000 rev./min. Higher speeds should be avoided because of the tendency to form gels which result in poor yields. The washed nuclei are collected *via* centrifugation at 1000 × *g* for 7 min. The wash procedure is repeated a second time using 1000 ml of 0.14 M NaCl in place of the Tris. The sedimented nuclei from this wash are resuspended to a final volume of 175 ml in 0.14 M NaCl, transferred to a one quart Mason jar, and 175 ml of 2.0 M NaCl, 0.03 M Tris (pH 7.5) added. The resulting gel is homogenized for 30 sec in a Polytron assembly mounted in a Mason jar cap (Bronwill Scientific) and driven on a Waring Blendor base running at top speed. After homogenization, 200 ml of 0.02 M Tris (pH 7.5) are added to the Mason jar, and the material is blended another 30 sec at top speed. The resulting viscous suspension is then transferred to a large beaker and 225 ml of 0.02 M Tris (pH 7.5) are added slowly while stirring by hand with a glass rod. Finally 100 ml of 0.02 M Tris (pH 7.5) are added quickly, resulting in the formation of a large gelatinous

precipitate of nucleohistone. (Under circumstances where degradation has been extensive, a distinct precipitate will not form at this point and the preparation should be discarded.) The precipitate should be broken up by blending in a standard Waring Blendor container at 1000 rev./min for 30 sec. The resulting suspension is centrifuged for 60 min in a Type-42 Spinco rotor at 42 000 rev./min (205 700 × *g*), and the supernatant is collected.

To the supernatant are added 10 g of Bio-Rex 70 (Na^+) which have previously been equilibrated with 0.4 M NaCl, 0.02 M Tris (pH 7.5). After stirring slowly for 10 min, the suspension is centrifuged at 6000 × *g* for 10 min and the supernatant withdrawn. The resin is washed by suspending it in 50 ml of 0.4 M NaCl, 0.02 M Tris (pH 7.5) and by centrifuging again for 10 min at 6000 × *g*. The two supernatants are then combined and 132 mg calcium phosphate gel[7] are added. After slow stirring for 20 min, the gel is centrifuged 5 min at 6000 × *g* and the supernatant discarded. The gel is washed by resuspension in 250 ml of 1.0 M $(NH_4)_2SO_4$, 0.05 M Tris (pH 7.5) using a motor-driven stirrer at 1000 rev./min, followed by centrifugation at 6000 × *g* for 5 min. The supernatant is again discarded, and the gel then dissolved in 8 ml of 0.3 M EDTA (pH 7.5), 0.33 M $(NH_4)_2SO_4$ by gentle hand homogenization with a Teflon Potter-Elvehjem tissue grinder. To maximize the amount of material dissolved, the suspension is allowed to stand for 1 h in the cold with occasional rehomogenization. The insoluble residue is then removed by centrifugation for 15 min at 33 000 × *g*, and the supernatant desalted by passing over a column of Bio-Gel P-10 (1.5 cm × 40 cm) equilibrated and eluted with 0.05 M Tris (pH 7.5). Larger columns may be employed when handling larger preparations. The exclusion peak contains the phosphoprotein and is pooled and stored frozen. The yield from this procedure is about 40 mg phosphoprotein per 8 g of nuclei, which represents a recovery of about 27%.

The above procedure includes several modifications of the method as originally reported, and the details should be carefully followed to ensure reproducible preparations. The present recovery of 27% is more than twice that obtained previously, indicating that these changes have significantly increased the yield as well as the reproducibility of the procedure. Aside from the minor changes in the volumes of solutions employed, the present method includes the following major changes: (1) increasing the concentration of nuclei in the 1.0 M NaCl extraction step about 50%; (2) employing a Mason jar Polytron assembly in the homogenization step; (3) omission of one centrifugation step prior to diluting the NaCl concentration to 0.4 M; (4) increasing the gravitational force in the centrifugation of the nucleoprotein precipitate; and (5) doubling the concentration of calcium phosphate gel used to adsorb the phosphoprotein. This last factor seems to be the most critical one in determining the yield of phosphoprotein. If the amount of calcium phosphate employed is increased even higher, more phosphoprotein can be recovered. However, contamination by other proteins increases at a faster rate than the yield of phosphoprotein, so the two factors must be carefully balanced against each other.

Another variable which has been altered is the volume and concentration of EDTA solution used to dissolve the calcium phosphate gel. It was found that by increasing the concentration of EDTA to 0.3 M and by decreasing the volume used several-fold, total recovery of phosphoprotein could be achieved in a much smaller volume. Although this factor does not seem to affect the total recovery, it does allow

for the processing of more phosphoprotein and thereby more preparations on a single Bio-Gel column.

In addition to the above factors, other variables have been examined in attempts to increase the yield of phosphoprotein. We have previously observed that spermidine is a natural component of the calf thymocyte nucleus, and its substitution for Ca^{2+} during nuclear isolation apparently confers greater stability on the nuclei without noticeable loss in nuclear function. In order to determine whether such a substitution would increase the yield or purity of phosphoprotein preparations, experiments were performed comparing the effects of Ca^{2+}, spermidine and spermine during nuclear isolation. These polyanions were also substituted for Mg^{2+} in the wash solution. It was found that the polyamines resulted in phosphoprotein preparations with lower yield and less purity than those obtained with Ca^{2+} and Mg^{2+}.

Since it is known that the concentration of divalent cations can affect nucleoprotein structure[8] and since polyamines, which are more efficient in binding to deoxynucleoprotein than divalent cations, actually reduced the yield of extractable phosphoprotein, experiments were performed to see if removing all metallic cations might improve the preparations. In these experiments, 2 mM EDTA was included in the procedure during all steps starting with the addition of 2.0 M NaCl. This concentration of EDTA was sufficient to chelate all the Mg^{2+} and Ca^{2+} known to be present in these nuclei[9]. The phosphoproteins obtained in these experiments were indistinguishable in terms of yield and purity from those prepared in the absence of EDTA.

Since the experiments of LANGAN[1] clearly demonstrated that phosphoprotein readily binds to histone, the possibility that losses in phosphoprotein were being incurred at the time of removal of these basic proteins with Bio-Rex 70 was also investigated. This was done by varying the amount of Bio-Rex added up to 10 times the normal amount. No change in the amount of protein adsorbed was observed, nor was it possible to further remove any protein by a second addition of Bio-Rex. Changes in the concentration of this resin appeared to have no effect on the purity or yield of the final phosphoprotein, and in many cases the resin treatment could be omitted entirely with no difference in results. The amount of resin used in the final procedure was selected to permit convenient removal by centrifugation, although less is actually required to adsorb any basic proteins present.

One striking problem in the study of the nuclear phosphoprotein fraction is its relative insolubility and lack of stability. Concentration of dilute phosphoprotein solutions could be achieved by dehydrating 4–5-ml aliquots in dialysis tubing with Sephadex G-200. By using this technique and by constantly mixing the solution, it is possible to reach a protein concentration of 1 mg/ml before the protein begins to precipitate. Precipitation at this point was observed even in the presence of 4–8 M urea, 1–5 M $LiCl_2$, and 10–40% guanidinium chloride. Neither treatment with deoxyribonuclease nor CsCl gradient centrifugation facilitated the handling of this material. Various detergents were also employed in an effort to improve the solubility of this preparation. Most were relatively ineffective, with the notable exception of Nonidet P-40 (Shell Chemical Co.).

Besides their relative insolubility, the nuclear phosphoproteins were also found to be unstable at 25° or even during extended dialysis at 4°. It was further found that the material, once precipitated, was all but impossible to resolubilize. The only

way found to resolubilize phosphoprotein previously precipitated with acetone was by treatment with 1% Nonidet P-40, which dissolved approx. 80% of the protein. Unfortunately, the use of this detergent presented another problem in that it could not be subsequently removed. It was found that prolonged dialysis or gel filtration with Sephadex G-25 did not remove sufficient amounts of the detergent to permit spectral analysis of the protein.

The difficulty in obtaining concentrated solutions of phosphoprotein almost precludes the possibility of routine fractionation procedures, such as electrophoresis and chromatography, and the problem of resolubilization makes techniques based on selective precipitation unfeasible. These qualities of this protein preparation must be kept in mind when considering the various approaches available for its handling and purification.

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