A Phosphate Staining Reagent Revisited

N. SANKAR and Dinkar SAHAL*,†
Department of Biophysics, University of Delhi South Campus, Benito Juarez Road, New Delhi-110021, India
(Received March 25, 1993)

A phosphate staining reagent [S. K. Goswami and C. F. Frey, *J. Liquid Res.*, **12**, 509 (1971)] was found to show differential staining of phosphate esters. Thus, the staining reaction mixtures in cases of molecules like dodecyl dihydrogenphosphate [DDP], phosvitin and phosphocellulose showed intense blue colors which on standing precipitated down leaving a colorless supernatant. However, at equimolar phosphate concentrations molecules like inorganic phosphate/diphosphate, ATP, and phosphoserine stained poorly. It was apparent that (a) the staining does not involve hydrolysis of phosphate esters and (b) the proximity between phosphate esters determines the staining intensity of different molecular systems. In order to confirm the proximity model, constant amounts of DDP were incubated with the staining reagent in the presence of increasing concentrations of sodium dodecyl sulfate [SDS]. A decrease in staining of phosphate was seen with progressive increase in the concentration of SDS. The slope of this inhibition curve was enhanced when the concentration of DDP was clamped at 300 μM (1 M=1 mol dm^{-3}) than was the case at 450 μM. The results of these experiments are suggestive of competitive binding of SDS to aggregates of DDP, a phenomenon which decreases the proximity relationship between phosphate head groups of DDP. This has provided us a new method for the estimation of SDS and amphiphiles of similar kind which have the ability to alter proximity relations in DDP aggregates. Phosvitin, a phosphoprotein rich in clusters of intramolecular phosphate esters was only marginally sensitive to the presence of SDS for its staining by this reagent.

Goswami and Frey[1] have described a molybdate based reagent that stains phospholipids but fails to stain phosphate esters like glycerol-2 and 3-phosphates or fructose-6-phosphate. The inherent suggestion is that the reagent may be specific for dialkyl phosphates. However, the precise reasons for this apparent selectivity have remained unknown for over two decades now. We show here that this reagent is capable of staining monoalkyl phosphates as well, that the chemistry of staining does not involve hydrolysis of phosphate esters and that close proximity between phosphate head groups is the factor determining the intensity of staining.

Experimental

The phosphate staining reagent[1] used here has been prepared by taking a mixture of 80 mg metallic copper and 250 mg ammonium molybdate in 1 ml of Mili Q water. The solution is chilled and 1 ml H_2SO_4 (concentrated 98%) added, the deep blue solution is shaken and kept for 2 h at 25—30 °C with occasional shaking. 40 ml Mili Q water is then added, the mixture upon shaking changes its color from deep blue to light brown. The copper metal is removed and 3.2 ml of concentrated H_2SO_4 is added, the resulting solution remains light brown. Goswami and Frey have used this as a qualitative spray reagent. In our study, this reagent (100 μl) has been added to test solution (900 μl), the mixture heated at 60 °C for 1 min and absorbance recorded at 754 nm after 1 min interval at 25 °C.

Dodecyl phosphate was synthesized by dropwise addition of dodecanol into $POCl_3$ using a method described separately.[2] Its melting point, ^1H NMR spectrum and mass spectrum were fully consistent with its structure.

†Present address: International Centre for Genetic Engineering & Biotechnology, Aruna Asaf Ali Marg, NII Campus, New Delhi-110067, India.

Results and Discussion

Figure 1 shows the results of staining of a wide spectrum of molecules (500 μM each) with the phosphate staining reagent of Goswami and Frey. Dodecyl dihydrogenphosphate (DPP) showed distinctly higher staining intensity. In contrast, serine, phosphoserine, inorganic phosphate, diphosphate, ATP, and sodium dodecyl sulfate (SDS) showed marginal or no staining at all. The inset to Fig. 1 shows the significantly higher staining of phosphoseryl phosphate esters in phosvitin than is the case with phosphoserine alone. Bovine serum albumin (not shown) was not stained by this reagent. The staining of phosvitin, phosphocellulose, and DDP showed blue pellets with colorless supernatants suggesting that the staining treatment used here does not cause hydrolysis of the phosphate esters. It is of particular interest to note that this stain of phosphate esters predicts intense staining for molecules with a cluster of intramolecular phosphate esters e.g. phosphocellulose and phosvitin or molecular organizations in which intermolecular interactions align phosphate esters into close proximity e.g. DDP. In a similar vein it predicts poor staining for inorganic phosphate and all those derivatives which tend to remain as monomers in solution e.g. phosphoamino acids, diphosphate, glucose-6-phosphate, ATP etc.

Figure 2 shows the results of staining of DDP in presence of increasing amounts of SDS. A significant reduction in absorbance is seen as a function of increasing concentration of SDS. When the DDP concentrations were clamped at 450 and 300 μM respectively, the slope of the curve was found to be higher in the latter than in the former case. This change in the value of slope upon a change in the clamp concentration of the alkyl phos-

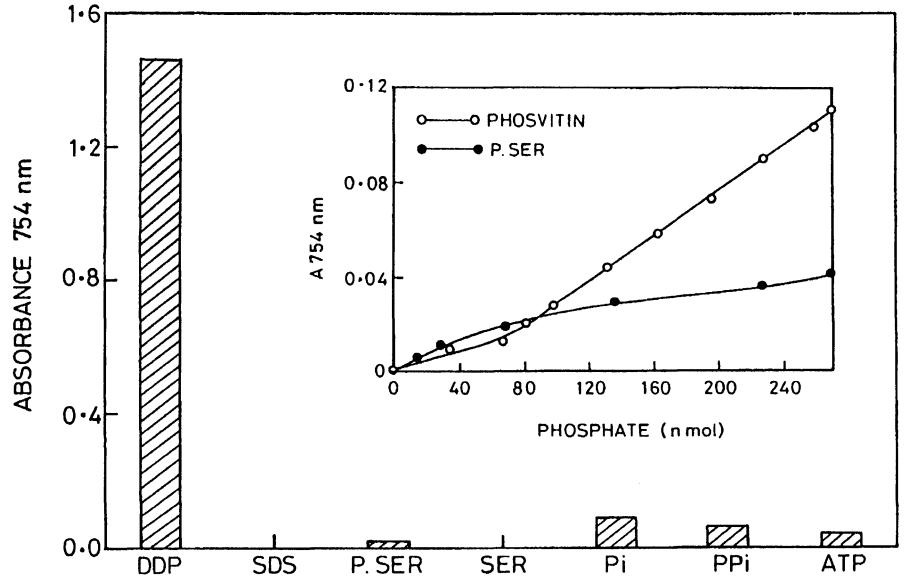

Fig. 1. Staining of the indicated molecules (500 μM each) with the phosphate staining reagent of Goswami and Frey.[1] Inset shows the staining characteristics of phosvitin (○) and phosphoserine (●), respectively. One μg phosvitin corresponds to 3.25 nmol of phosphate.

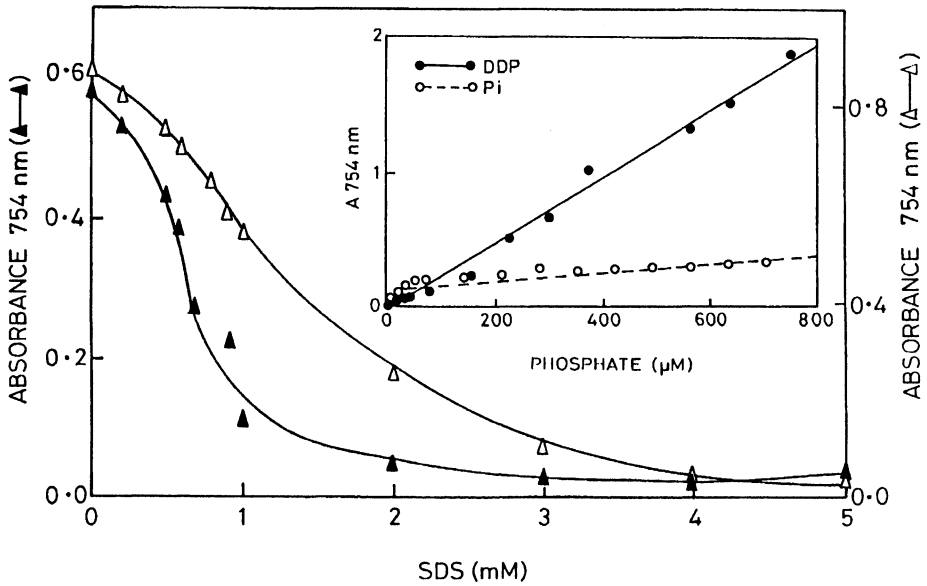

Fig. 2. Effect of competitive binding of SDS to DDP aggregates on the staining of DDP. DDP concentrations were clamped at 450 μM (△) and 300 μM (▲), respectively. Inset shows the concentration dependent staining curves of DDP (●—●) and inorganic phosphate (○- - -○), respectively.

phate suggests a competitive binding phenomenon in which SDS and DDP tend to make mixed micelles. The introduction of SDS into DDP micelles progressively decreases the proximity between phosphate esters. As is evident from the Experimental section, the staining reaction is done in 0.32 equiv H_2SO_4 (pH 0.8). At this pH not only DDP but also SDS is likely to be protonated and free of any negative charge. Hence it is unlikely that the observed SDS induced inhibition of the staining of DDP in mixed micelles is due to electrostatic repulsion between SDS and the anionic molybdate clusters. The inset in Fig. 2 shows the concentration dependent staining pattern of dodecyl phosphate and inorganic phosphate respectively. It is apparent that the amphiphilic phosphate detergents like DDP which aggregate in water show a concentration dependent increase in phosphate staining. This is clearly not the case with inorganic phosphate which exists as monomers in water.

In order to check that the inhibition in staining seen in presence of SDS is indeed caused by a competitive binding phenomenon, the effect of SDS on the staining characteristics of phosvitin was studied. Phosvitin was

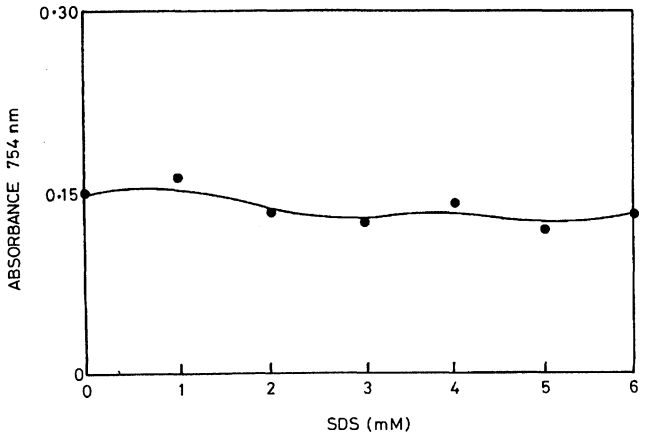

Fig. 3. Effect of SDS on the staining of phosvitin (150 μg).

chosen since it is a phosphoprotein in which clusters of contiguous phosphoserine residues are intramolecularly organized.[3,4] The molecular weight heterogeneity of phosvitin has been studied by the use of 0.1% (3.5 mM) SDS[5] suggesting that like all other proteins phosvitin binds SDS. However, the binding of SDS to phosvitin is not expected to alter the proximity interaction of its intramolecularly organized phosphate esters. So we predicted that SDS should not significantly alter the staining intensity of phosvitin by this reagent. As shown in Fig. 3, with increasing concentrations of SDS (0—6 mM) there was only a marginal decline in the staining intensity of phosvitin.

We have earlier reported that *N*-dodecanoylhistidine methyl ester has the ability to delay the appearance of phosphate staining of DDP by this reagent.[6] In this study ^{1}H NMR data was suggestive of ion pair interaction between *N*-dodecanoylhistidine methyl ester and DDP. Such an interaction will enhance the mutual distance between the phosphate esters. Our "proximity" model can therefore by hind sight predict that the alkyl imidazolium ion should indeed retard the staining of DDP. The exact nature of the chromophoric complex formed in this stain is not known. However, the role of mutual interactions between a cluster of proximally oriented phosphate esters and molybdate in generating an intense blue color is evident from our studies. Hence, additives which are capable of altering the proximity relationship between phosphate esters can conveniently be quantitated by this phosphate staining reagent.

The authors thank Ms. R. Radha for her help in preparation of this manuscript. N. S. is S. R. F. of the CSIR. This investigation was supported by CSIR grant 9(301)/90.

References

1) S. K. Goswami and C. F. Frey, *J. Lipid Res.*, **12**, 509 (1971).
2) A. K. Sinha, N. Sankar, J. Verma, and D. Sahal, submitted for publication.
3) F. J. Joubert and W. H. Cook, *Can. J. Biochem. Physiol.*, **36**, 399 (1958).
4) R. Shainkin and G. E. Perlmann, *J. Biol. Chem.*, **246**, 2278 (1971).
5) R. A. Wallace and J. P. Morgan, *Anal. Biochem.*, **157**, 256 (1986).
6) J. Verma and D. Sahal, *Bull. Chem. Soc. Jpn.*, **65**, 1719 (1992).