After equilibrating cells with \[^{32}\text{P}\]\text{phosphate} there were 3.7 ± 0.2 mol of \[^{32}\text{P}\]\text{phosphate/mol} of Band 2 subunit; this is the total measured as nonradioactive phosphate in fresh cells (Harris, H. W., Wolfe, L. C., and Lux, S. E. (1978) \textit{Fed. Proc.} 37, 1567). This level can be reduced in intact cells by metabolic depletion, or on purified spectrin by tryptic removal of the terminal phosphorylated peptides, or by treatment with bacterial alkaline phosphatase. Dephosphorylation by these methods did not change either the affinity or saturation level for rebinding of spectrin to high affinity sites on spectrin-depleted inside-out vesicles (10^−7 M and 125 μg/mg of membrane protein).

The \[^{32}\text{P}\]\text{phosphate} level was the same on spectrin extracted from membranes, residual membrane-bound spectrin, and on dimers and tetramers, suggesting that phosphorylation does not regulate the affinity of these binding interactions.

After removal of glucose from red cell suspensions, cell ATP drops to one-half its initial level in about 1 h, followed by crenation of half of the cells in 3 to 4 h; however, no detectable dephosphorylation of spectrin occurs until 4 to 7 h. In addition, no dephosphorylation was observed during crenation and deformability loss induced by the calcium ionophore, A23187.

We find no evidence for a causal relationship between spectrin phosphate levels and either red cell shape or spectrin binding to the membrane. These observations eliminate several simple mechanism by which spectrin phosphorylation-dephosphorylation might be involved in controlling cell membrane properties.

Phosphorylation of proteins in the erythrocyte membrane was first demonstrated in 1971 (1). Its function remains obscure in contrast to the well known regulatory significance of protein phosphorylation elsewhere (2). Spectrin (Bands 1 and 2) is the major extrinsic membrane protein of the red cell cytoskeleton and appears to be involved in determining cell shape and membrane deformability properties (4, 5). It is a multiply phosphorylated protein (6) and its dephosphorylation has been correlated with the creation induced by either metabolic ATP depletion (7) or incubation of erythrocytes with low density lipoprotein (8). Conversely, MgATP-dependent spectrin phosphorylation in crenated ghosts is correlated with the restoration of disc morphology (9, 10). One popular interpretation of these observations is that phosphorylation and dephosphorylation bring about significant changes in the physical properties of spectrin or its interactions with other membrane components thus causing changes in cell shape and mechanical properties.

It is known that erythrocytes must be metabolically active to maintain their disc shape (7) and deformability. During ATP depletion the viscosity of cell suspensions increases and membranes of single cells become less deformable (11). Changes are not due to ATP depletion \textit{per se}, but can also be brought about by increasing intracellular calcium levels (12) or by certain membrane intercalating drugs (13). Maintenance of cell shape is dependent on spectrin. The lipid component of ghosts can be extracted with nonionic detergents leaving a protein shell, primarily spectrin, which maintains the shape of the original cell, whether disc, sphere, echinocye, or sickle-shaped (14, 15). Spectrin can be extracted from ghosts into low ionic strength alkaline buffers (16), however, extractability is diminished in ghosts from ATP-depleted cells (17), suggesting an altered physical state of spectrin in crenated cells. During metabolic depletion, spectrin becomes dephosphorylated (18) and this alteration has been suggested as a cause for changes in membrane properties in cells (19) as well as in ghosts (10). However, our results show there is no causal relationship between spectrin phosphorylation and changes in cell shape or spectrin-binding interactions.

**EXPERIMENTAL PROCEDURES**

**Materials**

The following radioisotopes were purchased from New England Nuclear: \[^{32}\text{P}\]\text{orthophosphoric acid, carrier-free in 0.02 n HCl; \[^{32}\text{P}\]}\text{labeled Bolton-Hunter reagent, \[^{32}\text{P}\]}\text{iodinated p-hydroxyphenylpropiac acid N-hydroxysuccinimide ester} about 1500 Ci/mmol. Strep-tozymicin sulphate and penicillin G were from Nutritional Biochemicals. PEI-cellulose thin layer chromatography plates, Polygram CEL 300, were from Brinkmann Instruments Inc. Firefly lantern extract, FLE-50, and bacterial alkaline phosphatase Type III were from Sigma Chemical Co. Trypsin treated with L-1-tyosylamide-2-phenylethyl chloromethyl ketone was from Worthington Biochemical Corp. The calcium ionophore, A23187, was a gift from the Eli Lilly Co. All other chemicals used were of at least reagent grade.

**Methods**

\[^{32}\text{P}\]Phosphorylation and Dephosphorylation of Spectrin in Cells—Equilibration of human red blood cells with \[^{32}\text{P}\]phosphate was done by modifications of the methods of Bennett and Branton (20). Freshly drawn blood was washed several times by centrifugation (5 min, 3000 rpm, SS34 rotor) in 130 mM \text{NaCl, 3.7 mM KCl, 2.4 mM MgCl}_2, 1.2 mM CaCl}_2, 25 mM NaHCO}_3, pH 7.5 (Buffer A), the buffy coat removed and the red cells suspended at a 20% hematocrit in Buffer A plus 1 mM adenosine, 10 mM glucose, 0.1 mg/ml of both streptomycin and penicillin G, and 0.4 to 0.5 mCi of neutralized
NaH$_2$P$_4$O$_6$ of packed cells. Incubation was done in a capped plastic tube at 37°C with mild shaking. At the indicated times, $[^{32}P]$spectrin was either extracted for studies described below or was metabolically dephosphorylated in cells by washing adenosine and glucose away and continuing the incubation at 37°C. Five washes, each with 10 volumes of Buffer A at 0–4°C were used. Over a period of 15 min in the ultracentrifuge, the pellet dropped by 2.3%. No cell lysis was detected with a hemocytometer although a slight red color was sometimes seen in the first postein supernatants.

**Purification of Spectrin—**Published methods (20) were used for preparation for erythrocyte ghosts and extraction of spectrin into 0.3 mM Na$_2$PO$_4$, pH 7.6 for 10 min at 37°C. Extracted membranes were pelleted by centrifugation (100,000 × g 15 min) and the spectrin dimer purified from tetrarmers and other proteins by velocity sedimentation on 12-m1 linear sucrose gradients (5 to 20%). Gradient buffer was modified to contain 0.1 mM ethylenediaminetetraacetic acid (EDTA), but no MgCl$_2$ or ATP. Spectrin tetrarmers and dimers were also fractionated from whole extracts by gel filtration on Bio-Rad Bio-Gel A-15m column (1.5 × 60 cm) with 150 mM NaCl, 20 mM Tris, pH 8.0, and 1 mM EDTA at 4°C as the eluant.

**Determination of Covalent $[^{32}P]$Phosphate on Band 2—**This measurement required determination of the $[^{32}P]$spectrin specific radioactivity on Band 2. $[^{32}P]$ATP specific radioactivity and that of other adenine nucleotides was measured by the method of Gonzalez and Gee (21) adapted to fractionated from whole extracts by gel filtration on Bio-Rad Bio-Gel A-15m column (1.5 × 60 cm) with 150 mM NaCl, 20 mM Tris, pH 8.0, and 1 mM EDTA at 4°C as the eluant.

**Location of Bound Phosphate—**All $[^{32}P]$PO$_4$ on spectrin was determined by the method of Lowry (28) using bovine serum albumin as standard. Native spectrin and tryptic concentrations were determined by absorbance at 280 nm assuming an E$_{1%}$ of 10.1 (29) and 14.3 (30), respectively. Preparative and analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed on slab gels as described by Davies and Stark (31) and UV irradiation. Coomassie blue-stained gels were dried on filter paper and radioautographed on Kodak NS-2T x-ray film. Low ionic strength extracts of $[^{32}P]$PO$_4$-labeled ghosts were iodinated by the method of Bolton and Hunter (32) and purified on sucrose gradients as described above. Simultaneous counting of radioactivity from $[^{32}P]$PO$_4$ and $[^{35}S]$PO$_4$ was done in 5 ml of Aquasol correcting for overlap of emission spectra by standard methods.

**RESULTS AND DISCUSSION**

**Location of Bound Phosphate—**All $[^{32}P]$PO$_4$ on spectrin is bound within less than 8,000 daltons (25) of one terminal (33) of Band 2. Mild trypsinization of native spectrin produces a fragment from Band 2, about 8,000 daltons less than Band 2, containing no phosphate (Fig. 1B). When endogenously $[^{32}P]$phosphorylated and $[^{35}S]$-labeled Bolton-Hunter reagent-iodinated spectrin is trypsinized (Fig. 1A) and fractionated on sucrose density gradients by velocity sedimentation (Fig. 2), the terminal phosphorylated regions of Band 2 remain near the top of the gradient leaving the bulk of the protein with an unchanged sedimentation value. The phosphorylated peptides partition on gel chromatography columns under nonnaturating conditions as two peaks both smaller than cytochrome c (data not shown). These phosphorylated regions may constitute two small domains readily separated by proteolysis from the rest of the protein.

**Quantification of Spectrin Phosphate—**Use of $[^{32}P]$ATP specific radioactivity to quantify $[^{32}P]$PO$_4$ on spectrin requires that the spectrin-bound $[^{32}P]$phosphate has reached a steady state so that all exchangeable sites are in isotopic equilibrium. Further the α, β, and γ-phosphates of ATP must reach some known equilibrium. Under our incubation conditions, the specific radioactivity of spectrin reached a steady state between 15 and 18 h; the total ATP concentration remained constant up to the longest times measured, 30 h. The specific radioactivities for ATP, ADP, and AMP after 24-h equilibration, averaged over four cell incubations, were in a ratio of 3.2:0.1:0.96. Therefore it was assumed that the α, β, and γ positions of phosphates were in equilibrium and the specific radioactivity of the phosphate was taken to be the third of that of ATP.

The average number of exchangeable covalent phosphates per Band 2 polypeptide is 3.7 ± 0.2. This number is the same 2 Standard deviation = (2σ$^2$/N − 1)$^{1/2}$. Four independent 24-h incubations were done, as described under "Methods."
as that determined as nonradioactive phosphate in spectrin from fresh blood (6). Thus the exchangeable [32P]P04 incorporated under our in vitro conditions is the same as the number of total covalent phosphates. Until the total number of potentially phosphorylated sites is known, it remains a question whether this represents essentially full occupancy of four sites per Band 2 or partial occupancy of a greater number of sites. In this paper, fully phosphorylated spectrin refers to spectrin preparations containing approximately 3.7 mol of phosphate/mol of Band 2.

Others have reported a diminished spectrin extractability at 0°C in ATP-depleted cells (17). If phosphorylation permits extractability, then extractable spectrin would be expected to have a higher [32P]PO4 specific radioactivity than spectrin on whole ghosts or residual nonextractable spectrin. The extraction procedure described here releases approximately 80% of the spectrin, 20% remains with the pelleted membranes. Both species have the same specific radioactivity (Table I). This is true of both fully phosphorylated and 40% dephosphorylated spectrin, from normal and crenated cells, respectively. Spectrin in these extracts may be associated as dimers, as tetramers or with Band 4.1 and red cell actin (4, 35). When extracts are fractionated by gel filtration on Bio-Gel A-15m approximately 70% of the spectrin elutes as dimers, 20% as tetramers, and 10% is excluded in the void volume along with small amounts of Band 4.1 and actin. The phosphate content of these species is the same indicating that these interactions are probably not influenced by phosphorylation (Table I). This is compatible with previous work demonstrating an unaltered dimer-tetramer equilibrium after alkaline phosphatase treatment (36).

**Metabolic ATP Depletion, Red Cell Shape, and Spectrin Dephosphorylation**—If spectrin dephosphorylation causes cell crenation or is required before crenation can occur, it should precede or be coincident with this change. Representative results of experiments to test this hypothesis are presented in Fig. 3. After equilibrating metabolically active cells with [32P]PO4 for 25 h, the glucose was removed (arrow down at 0 h) and metabolic depletion allowed to occur. ATP levels fell rapidly reaching half the initial value by 1 h and being too low to measure by 6 h. Loss of disc shape lagged behind ATP loss; discocytes plus type I echinocytes declined to 50% at 3/2 h and crenation was complete at 12 h depletion. We could not reliably distinguish discocytes from type I echinocytes by light microscopy and so counted them together. This gives a very conservative measurement of shape loss and probably accounts for the paradoxical return to initial shape preceding return of ATP during ATP repletion. Photomicrographs of cells from the experiment depicted in Fig. 3 are shown in Fig. 4. The morphology of cells before ATP depletion is shown in Fig. 1a and after 2 and 12 h of depletion in Panels b and c, respectively. In contrast to an immediate decline in ATP and loss of disc shape after glucose removal, spectrin phosphate did not decline until after 6 h and then declined only slowly. This lag before dephosphorylation (4 to 7 h) may be due to slow dephosphorylation of some metabolite inhibiting the spectrin phosphatase. In fact, physiological concentrations of a variety of phosphorylated metabolites have been shown to inhibit
completely the cytosolic spectrin phosphatase under cell-free assay conditions (37) raising the possibility that phosphorylation is not an acutely regulated process in normal cells. Thus, crenation of metabolically depleted cells does not require spectrin dephosphorylation. This is also true of the crenation of spectrin-depleted inside-out membrane vesicles after dephosphorylation. This is also true of the crenation restoration experiment depicted in Fig. 3. a, 0 h just prior to depletion; b, 2-h depletion; c, 12-h depletion; d, 2-h repletion following 12-h depletion. Bar represents 5 μm.

V% 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59 61 63 65 67 69 71 73 75 77 79 81 83 85 87 89 91 93 95 97 99 10 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98 100 FIG. 4. Phase-contrast photomicrographs of representative cells at various times during metabolic ATP depletion experiment depicted in Fig. 3. a, 0 h just prior to depletion; b, 2-h depletion; c, 12-h depletion; d, 2-h repletion following 12-h depletion. Bar represents 5 μm.

Spectrin Phosphate and Red Cell Membrane Binding—Cell shape and deformability might be controlled by regulating the affinity of spectrin to spectrin-depleted inside-out membrane vesicles after dephosphorylation of the spectrin in three different ways. Dephosphorylated [32P]spectrin from metabolically depleted crenated cells bound to inverted vesicles with the same apparent dissociation constant and saturation binding level as fully phosphorylated spectrin (Kd = 41 μg/ml, 10^{-7} μM, and n = 125 μg/mg of membrane protein, Fig. 5). This high affinity binding has been shown to be to Band 2.1 on the membrane (38, 39). Similar results were obtained with spectrin dephosphorylated to levels of 45% and 23% and rephosphorylated from a level of 18% to 28% by addition of glucose to previously depleted cells. Even though these spectrin preparations were from thor-
Functional Role of Spectrin Phosphorylation

2. Fig. 6 shows the results of acrylamide gel electrophoresis of fully phosphorylated and alkaline phosphatase-treated spectrin after purification by velocity sedimentation. The binding parameters for fully phosphorylated dimer were independently established as $K_D = 10^{-7} \text{M}$, $n = 130 \mu\text{g/mg}$, and then the binding of this material in the presence of varying concentrations of dephosphorylated dimer was measured. A correction for residual radioactivity of 2% was not made. The experimental points for $[^{32}\text{P}]$spectrin binding fit well to a theoretical line which assumes an unchanged $K_D$ and $n$ for the dephosphorylated species (Fig. 7).

Finally, we purified the dimers after tryptic removal of 18% of their phosphate and found membrane-binding activity unaltered (Figs. 8 and 1A) (40). Velocity and sedimentation profiles of trypsinized and untrypsinized material are shown in Fig. 2. This result suggests that not only is the high affinity binding of spectrin to Band 2.1 on the membrane independent of spectrin phosphate level, but the binding site is not within that region proteolytically removed.

We attempted to isolate a proteolytic fragment with membrane-binding activity; however, using either trypsin or chymotrypsin, membrane binding was destroyed before the 7.6 s w, w, material was changed into discrete fragments.

Conclusion—The biological significance of spectrin phosphorylation remains an enigma. We have presented evidence that total phosphate levels on spectrin isolated in several functionally different association states are invariant and the association of spectrin with itself and with other membrane proteins is not controlled by phosphate levels. Binding characteristics of spectrin for its high affinity binding site on Band 2.1 of the membrane are idiosyncratic to phosphate levels. Preliminary experiments indicate the same is true of binding...
to Band 4.1 measured in velocity sedimentation gradients. Band 4.1 can be isolated in complexes with spectrin from low ionic strength extracts (35) and may provide additional lower affinity binding sites. Binding to actin, Band 5, is another reported spectrin interaction. Despite early reports that phosphorylation promotes spectrin's ability to induce actin polymerization (41) this remains controversial in light of conflicting results from two other laboratories (42, 43). Taken together these observations do not support phosphorylation-controlled equilibria of cross-links in the cytoskeletal meshwork as a mechanism of controlling membrane deformability. Finally, dephosphorylation is not required for crenation which accompanies in vitro metabolic depletion or ionophore-induced calcium entry. However, other mechanisms may operate in the blood to decrease spectrin phosphate levels, with functional significance, in the presence of normal ATP levels.

No in vitro parameter of spectrin function measured here is sensitive to phosphate levels; perhaps clues about a possible functional role might come from in vitro studies. For example, does spectrin phosphorylation vary with cell age, during erythroid development or aging, or in hemolytic anemias showing altered red cell morphology? Another possible mechanism for control of cell shape and deformability is by alterations of intracellular calcium. Calcium levels increase during ATP depletion (11) and ionophore A23187-induced crenation (12). Addition of calcium to ghosts causes an echinocyte-like spec- 

ification (44). Further, a decreased Ca²⁺ ATPase activity and elevated internal calcium have been reported in red cells of patients with hereditary spherocytosis (45, 46) in contrast to apparently normal phosphorylation of spectrin (22).

Relating total phosphate to function, as we have done, may be misguided if the critical variable is the site of phosphorylation rather than the extent. Such correlations, however, require more detailed chemical knowledge than is now available.

Acknowledgments—We thank Doctors G. Guidotti, H. W. Harris, and D. Branton for invaluable discussions and advice in the preparation of the manuscript.

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