Biochimica et Biophysica Acta, 470 (1977) 70—83

BBA 77801

THE PREPARATION AND USE OF PYRIDOXAL [^{32}P]PHOSPHATE AS A LABELING REAGENT FOR PROTEINS ON THE OUTER SURFACE OF MEMBRANES

RICHARD EGER and DANIEL B. RIFKIN

Department of Chemical Biology, Rockefeller University, New York, N.Y. 10021 (U.S.A.)

(Received February 25th, 1977)

Summary

Pyridoxal [^{32}P]phosphate was prepared using [γ-^{32}P]ATP, pyridoxal, and pyridoxine kinase purified from *Escherichia coli* B. The pyridoxal [^{32}P]phosphate obtained had a specific activity of at least 1 Ci/mmol. This reagent was used to label intact influenza virus, red blood cells, and both normal and transformed chick embryo fibroblasts. The cell or virus to be labeled was incubated with pyridoxal [^{32}P]phosphate. The Schiff base formed between pyridoxal [^{32}P]phosphate and protein amino groups was reduced with $NaBH_4$. The distribution of pyridoxal [^{32}P]phosphate in cell membrane or virus envelope proteins was visualized by autoradiography of the proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The labeling of the proteins of both influenza and chick cells appeared to be limited exclusively to those on the external surface of the virus or plasma membrane. With intact red blood cells the major portion of the probe was bound by external proteins, but a small amount of label was found associated with the internal proteins spectrin and hemoglobin.

Introduction

A number of membrane-impermeable agents, both anionic and cationic, have been used to label proteins on the external surface of membrane vesicles [1—14]. These probes have been designed to react with either the nucleophilic or the electrophilic groups of proteins on the membrane surface and by introducing a radioisotope via the bond formed between the labeling reagent and the membrane component. The charge or size of the labeling agent prevents its pas-

Abbreviations: PMSF, *p*-toluenesulfonyl fluoride; SDS, sodium dodecyl sulfate; PAS, periodic acid Schiff base; TEMED, N,N,N^1,N^1-tetramethylethylenediamine.

sage through the membrane and thus proteins in the interior of the vesicle are not labeled. The distribution of proteins in erythrocytes and other membranes has been studied in this way.

We have described the use of pyridoxal phosphate as a reagent for labeling proteins on the outer surfaces of membranes [7]. In this reaction the membrane vesicle is incubated with pyridoxal-*P* to allow the formation of a Schiff base, which is subsequently reduced by the addition of NaB^3H_4. The reduction of the Schiff base formed between pyridoxal-*P* and a protein amino group by NaB^3H_4 introduces ^3H into a stable covalent bond. Proteins that have reacted with pyridoxal-*P* can be separated by a technique such as acrylamide gel electrophoresis, and then identified by the ^3H associated with them. This reaction has been successfully applied to enveloped viruses, erythrocytes, and cultured animal cells.

Despite the effectiveness of the original procedure, the use of pyridoxal [^{32}P]phosphate appeared advantageous for several reasons. First, the use of pyrodoxal [^{32}P]phosphate would permit the visualization of derivatized protein by autoradiography of polyacrylamide gels rather than by scintillation counting of a sliced gel, with a significant increase in sensitivity and convenience. Second, since membranes are permeable to NaB^3H_4 any internal, naturally occurring Schiff bases are labeled even in the absence of pyridoxal-*P* [7]. The use of pyridoxal [^{32}P]phosphate would eliminate this background radioactivity since the radioisotope would not penetrate the membrane, and reduction of the external Schiff base could be performed with $NaBH_4$. Third, the availability of pyridoxal [^{32}P]phosphate would permit double label experiments to be performed using ^{32}P and ^3H.

We describe here a method for the preparation of pyridoxal [^{32}P]phosphate using pyridoxine kinase from *Escherichi coli*, [γ-^{32}P]ATP, and pyridoxal. This reagent, pyridoxal [^{32}P]phosphate, is effective in labeling the outer membrane proteins of influenza virus, red blood cells, and chicken embryo fibroblasts.

Materials and Methods

E. coli B, "three-fourths grown" in enrichment medium, was purchased from Grain Processing Corp., Muscatine, Iowa. Pyridoxal-HCl and pyridoxal 5′-phosphate were purchased from Calbiochem. Enzyme grade ammonium sulfate was purchased from Mann Research Laboratories. DEAE-cellulose was purchased from Whatman. Sephadex G-100, QAE, and DEAE-Sephadex A-25 were purchased from Pharmacia Inc. Hydroxyapatite was purchased from Biorad. Carrier-free [^{32}P]phosphate was bought from Schwarz/Mann. Aquasol was purchased from New England Nuclear. $NaBH_4$ was purchased from Fisher Scientific. SDS was purchased from British Drug Houses, PMSF from Aldrich, HEPES from Sigma, sodium metabisulfite from Matheson, Coleman and Bell, and Basic Fuchsin from Fisher Scientific. Aldolase, 3-phosphoglycerate kinase, and glyceraldehyde phosphate dehydrogenase were purchased from Boehringer Mannheim. Reagents for acrylamide gels were purchased from Eastman. All other chemicals used were the best commercial grade available.

Preparation of pyridoxine kinase. Pyridoxine kinase was prepared from *E. coli* essentially according to the method of White and Dempsey [15,16] with

the following modifications of the indicated steps.

The *E. coli* B were disrupted by grinding in a ceramic mortar with alumina A-305. The mixture was then centrifuged in Sorvall RC 2B centrifuge for 3 h at 25000 × *g*. The crude supernatant was decanted and diluted with 1.5 l of 10 mM potassium phosphate, pH 7.0, per 260 g bacteria. This solution was treated at 4°C with 0.5 ml of 1 M $MgCl_2$ per 100 ml of extract with stirring for 6—8 h. The mixture was then centrifuged for 1 h at 25000 × *g*. The precipitate was discarded.

Ammonium sulfate precipitation of the supernatant solution and DEAE chromatography using 80 mM KCl, followed by 120 mM KCl, pH 7.0, were performed essentially as originally described. The active fractions from the DEAE column were pooled and directly applied to a Sephadex QAE A-25 column (4 × 47 cm) equilibrated with 0.1 M KCl in 10 mM potassium phosphate, pH 7.0. After the first protein peak was eluted by the equilibrating buffer, a linear gradient of 0.1 M KCl (600 ml) to 0.18 M KCl (600 ml) in 10 mM potassium phosphate (pH 7.0) was used to elute the pyridoxine kinase. The most active fractions were pooled and concentrated by either of two procedures. The pooled fractions were dialyzed against 10 mM potassium phosphate (pH 7.0), applied to a small DEAE-cellulose column (0.9 × 4 cm), and eluted with 0.1 M KCl in 10 mM potassium phosphate buffer.

The concentrated enzyme was applied to a Sephadex G-100 column (4 × 130 cm) equilibrated with 10^{-5} M pyridoxal and 10^{-4} M $ZnSO_4$ in 10 mM potassium phosphate (pH 7.0) and eluted with this buffer. The fractions of highest activity were pooled and directly applied to a small DEAE-cellulose column equilibrated with the same buffer. The pyridoxine kinase was then eluted with a linear gradient six times the column volume consisting of 0—0.1 M KCl in the equilibrating buffer. The active fractions were pooled and dialyzed against 10 mM potassium phosphate buffer (pH 7.0) and then applied to a hydroxyapatite column (10 ml bed volume). The enzyme was then eluted with a 400 ml linear gradient of 0.01—0.3 M potassium phosphate, pH 7.0. The active fractions were dialyzed against 10 mM potassium phosphate, pH 7.0.

The enzyme was stored at —20°C. There was no detectable loss of activity after periods of storage as long as 8 months at —20°C.

The recoveries and specific activities of the various fractions are illustrated in Table I. At the final step in the purification procedure, the enzyme had been purified 1300-fold with a yield of 40% based on the initial activity in the bacterial extract. Electrophoresis of this material in SDS polyacrylamide gels demonstrated that the preparation was not yet homogenous. However, assays for phosphatase activity indicated that the enzyme preparation contained less than 10^{-7} units of alkaline phosphatase activity per ml. Moreover, the absence of any inorganic [^{32}P]phosphate formed during the incubation reaction of [γ-^{32}P]ATP, pyridoxal, and enzyme (vide infra) also indicated that the enzyme preparation contained no detectable phosphatase activity under the conditions used. Therefore, the enzyme at this stage could be employed for the synthesis of pyridoxal-*P* without further purification.

Apotryptophanase assay of pyridoxine kinase. Pyridoxine kinase was assayed by the apotryptophanase assay described by White and Dempsey [15,16]. The Crooks strain of *E. coli* was generously supplied by Dr. W.B. Demspey and was

used for the production of apotryptophanase.

Alkaline phosphatase. The assay used was that of Garen and Leventhal [17].

Synthesis of [γ-^{32}P]ATP. [γ-^{32}P]ATP was synthesized from carrier-free [^{32}P]phosphate (Schwarz/Mann) and ATP using the procedure of Schendel and Wells [18] with the following modifications. Glyceraldehyde 3-phosphate was generated enzymatically by including $2.5 \cdot 10^{-3}$ M fructose-1,6-diphosphate in the reaction mix. Six I.U. of aldolase were added with the 3-phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase to initiate the reaction. The total reaction mixture had a volume of 1 ml, and was incubated in the dark at 10°C. The synthesis of [γ-^{32}P]ATP was monitored by spotting 1-μl aliquots on a polyethyleneimine cellulose sheet and developing the thin layer with 1 M LiCl [17]. The purification of the [γ-^{32}P]ATP was done on a DEAE-Sephadex A-25 column as described by Schendel and Wells [18]. The yield of [γ-^{32}P]-ATP based on the amount of [^{32}P]phosphate initially used was 80—90%.

Synthesis of pyridoxal [^{32}P]phosphate. The synthesis of pyridoxal [^{32}P]-phosphate from pyridoxal and [γ-^{32}P]ATP by pyridoxine kinase was performed using the conditions described by White and Dempsey [15,16], with the following modifications. The various components of the reaction mixture were added directly to a plastic tube in which the [γ-^{32}P]ATP had been previously lyophilized. The components of the mix were first incubated in the dark for 15 min at 37°C. After this time, 0.02 unit of pyridoxine kinase in 2 ml of 10 mM potassium phosphate (pH 7.0) were added, and the mixture was shaken gently for 30 min in the dark at 37°C. The reaction was terminated by the addition of EDTA to a final concentration of 0.01 M. In addition, 20 μl of 10 mM pyridoxal-*P* was then added to act as a spectral marker.

Purification of pyridoxal [^{32}P]phosphate. The reaction mixture was diluted 20—30-fold with glass-distilled water and applied to a 2—3 ml column of DEAE-Sephadex A-25 that had been pretreated with ADP, washed with 0.4 M NH_4HCO_3 and re-equilibrated with 10 mM NH_4HCO_3. After the diluted reaction mixture had been applied to the column in the dark, the column was

TABLE I

PURIFICATION OF PYRIDOXINE KINASE FROM *E. COLI* B

Units of kinase activity are expressed as μmol of pyridoxal 5'-phosphate formed per min during a 15 min incubation of the enzyme with 0.1 mM ATP, 0.1 mM pyridoxal-HCl, 1 mM $ZnSO_4$ and 10 mM potassium phosphate buffer (pH 7.0). The reaction was stopped by boiling the samples for 5 min and the pyridoxal 5'-phosphate subsequently determined by either the apotryptophanase assay, pH 7.0, or the direct spectral assay at pH 6.0 as described in Materials and Methods.

Fractionation stage	Total units	Total protein (mg)	Volume (ml)	Specific activity $\times 10^3$	Yield (%)
1 Crude extract	0.826	24 078	967	0.034	100
2 MnCl trated (supernatant)	0.819	20 924	987	0.039	99.2
3 30—52% $(NH_4)_2SO_4$ precipitate	0.839	8 478	321	0.099	101.0
4 DEAE-cellulose, pH 7.0, stepwise	0.699	348	480	2.01	84.7
5 QAE-Sephadex A-25 gradient	0.551	49	610 → 43	11.24	66.7
6 Sephadex G-100 column	0.490	16.1	150	30.5	59.4
7 DEAE-cellulose, pH 7.0, gradient	0.426	11.3	75	37.8	51.6
8 Hydroxyapatite (HTP) column	0.325	7.2	33.3	45.0	39.4

washed with equilibrating buffer until the amount of radioactivity eluted was at background level. Consecutive elution with 0.1, 0.18, 0.23, and 0.4 M NH_4HCO_3 buffers was used and approx. 20-column volumes were collected at each step. The column fractions were assayed for radioactivity by pipetting 5 μl into 5 ml of Aquasol and counting the solution in a Packard Tricarb liquid scintillation counter. In addition, the absorbance of each fraction at 388 nm was determined to identify the pyridoxal-*P* fraction. Those fractions that showed both radioactivity and adsorption at 388 nm were pooled and concentrated by lyophilization. The purity of the pyridoxal [^{32}P]phosphate was assessed by thin-layer chromatography using two different chromatographic systems (methanol/benzene/γ-butanol/water/triethylamine (4 : 2 : 2 : 2 : 1, v/v) on polyethyleneimine cellulose sheets, and methylethylketone/water/ethanol/NH_3 (15 : 5 : 5 : 5, v/v) on silica gel G sheets) followed by autoradiography.

Preparation of virus and cells. WSN strain of influenza virus was grown in bovine kidney cells and purified as described previously [7]. Human red blood cells and erythrocyte ghosts were prepared from freshly drawn blood according to the methods described by Fairbanks et al. [21]. Chick embryo fibroblasts and Rous sarcoma virus (subgroup A) transformed chick embryo fibroblasts were prepared as described earlier [22].

Labeling reactions. Influenza virus. Purified influenza virus was suspended in 10 mM potassium phosphate (pH 7.0) at a concentration of 0.95 mg/ml in a plastic tube. To 1.95 ml of virus solution was added 1.25 ml of resuspended pyridoxal [^{32}P]phosphate (1.23 Ci/mmol; 234 μCi) in 50 mM HEPES buffer, pH 7.4. The virus-pyridoxal [^{32}P]phosphate mixture was incubated at 37°C in the dark. After 20 min, 20 μl of 0.1 M $NaBH_4$ dissolved in 0.01 M NaOH was added. The solution was mixed and placed on ice for 10 min. An excess of pyridoxal-*P* dissolved in 10 mM sodium phosphate was added dropwise until a yellow color persisted.

Virus was repurified and concentrated from the reaction mixture as described earlier. The virus pellet was resuspended in approx. 100 μl of 1% sodium dodecyl sulfate (SDS). Protein concentration was determined and suitable aliquots of the pyridoxal [^{32}P]phosphate-labeled virus were subjected to electrophoresis on 10% polyacrylamide slab gels containing SDS. The acrylamide gel was stained with 0.2% Coomassie Brilliant Blue dissolved in 50% trichloroacetic acid, destained in 7% acetic acid, and dried. The distribution pyridoxal [^{32}P]phosphate protein was determined by autoradiography, and the developed autoradiographs were scanned with a Joyce-Loebl densitometer.

Erythrocyte labeling. Human erythrocytes were obtained from fresh blood according to the procedure of Fairbanks et al. [21]. Ghosts were isolated after hypotonic lysis of the washed erythrocytes [21].

For labeling experiments, 0.25 ml of packed red blood cells or packed ghosts were mixed with 100 μl of freshly synthesized pyridoxal [^{32}P]phosphate (0.54 Ci/mmol; 38 μCi) dissolved in Dodge's buffer [23]. In addition, 0.5 μl of 2.0 M PMSF was added. The reaction mixture was incubated for 30 min at 37°C in the dark. At the end of this period, the tubes were placed on ice, and two 5-μl aliquots of 0.1 M $NaBH_4$ in 10 mM NaOH were added at 5-min intervals. Excess pyridoxal-*P* was then added until a yellow color persisted. The red blood cells were gently washed six times with isotonic buffer and isolated by centrifuga-

TABLE II

COMPOSITION OF POLYACRYLAMIDE GELS

	Stacking gel	Polyacrylamide	Polyacrylamide	Polyacrylamide	
	(ml)	(8%)	(10%)	6%	16%
Stock acrylamide	1	6.4	8.0	4.8	12.8
Water	6.5	11.6	10.0	13.2	2.4
Upper gel buffer	2.5	—	—	—	—
Lower gel buffer	—	6.0	6.0	6.0	6.0
$(NH_4)_2S_2O_8$	0.3	0.05	0.05	0.05	0.05
TEMED	0.01	0.12	0.12	0.12	0.12
Sucrose, 65%	—	—	—	—	2.8

tion at 1700 × *g*. The labeled erythrocytes were lysed by the addition of 14 ml of 5 mM sodium phosphate, pH 8.0. The ghosts were isolated by centrifugation at 16000 rev./min for 10 min in a Sorvall SS-34 rotor. The cell lysate was saved for further analysis. The radioactively labeled ghosts were also repurified from excess pyridoxal [^{32}P]phosphate and other reaction products as described above.

The distribution of pyridoxal [^{32}P]phosphate in the proteins of erythrocytes and ghosts was determined after SDS polyacrylamide gel electrophoresis of the samples on slab gels of 6—16% acrylamide gradients. Proteins were detected by staining the gels in 0.2% Coomassie Blue dissolved in 50% trichloroacetic acid. Destaining was performed in 7% acetic acid. In order to detect carbohydrates, adjacent wells containing the same material were stained by the PAS reaction [24]. The gel was fixed for at least 1 h in 12.5% trichloroacetic acid, briefly washed twice with distilled water and the water carefully removed. The gel was then incubated for 1 h in freshly prepared 1% periodic acid in 3% acetic acid at 37°C. After 4—5 washes with distilled water during a 5 h period at 37°C, the gel was incubated with Schiff reagent for 1 h at 37°C in a covered dish. The Schiff reagent was prepared by mixing 8 g of basic Fuchsin, 16 g of sodium metabisulfite, and 21 ml of concentrated HCl. This mixture was stirred for 2 h at room temperature with activated charcoal, filtered, and stored at 0—4°C. After incubation with the Schiff reagent, the gel was washed repeatedly with 0.5% sodium metabisulfite.

The stained gels were then dried and pyridoxal [^{32}P]phosphate was detected by autoradiography. The exposed X-ray film was analyzed by densitometry using a Joyce-Loebl densitometer.

Labeling of Chick Embryo Fibroblasts and Rous sarcoma virus-transformed chick embryo fibroblasts. One 60-mm plate (10^6 cells) each of chick embryo fibroblasts and Rous sarcoma virus-transformed chick embryo fibroblasts was washed twice with 5 ml of buffer A (0.15 M NaCl, 0.02 M sodium phosphate, pH 8.0). 1 ml of this buffer containing pyridoxal [^{32}P]phosphate (6.2 Ci/mmol; 138 μCi) was added to each plate and the cells were incubated at 4°C in the dark for 15 min. The Schiff bases were reduced by the addition of 50 μl of 50 mM $NaBH_4$ dissolved in buffer A. The plates were gently swirled and left at 4°C for 10 min. Additional pyridoxal-*P* dissolved in buffer A was then added to

each plate until a yellow color persisted. The cells were carefully scraped from the dish with a rubber policeman, collected by centrifugation at 1500 rev./min in an International PR6 centrifuge, and gently washed five times in 10 ml of buffer A. Each cell pellet was dissolved in 200 μl of 1% SDS. Protein was determined by the method of Lowry et al. [25]. The proteins in both normal and transformed cells were analyzed by SDS polyacrylamide gel electrophoresis, followed by staining with Coomassie Blue. The incorporation of pyridoxal [^{32}P]phosphate was visualized by autoradiography of the dried gels.

Polyacrylamide gels. Essentially, the technique described by Laemmli [26] was used in a slab apparatus modified after the one described by Studier [27].

Results

Synthesis of pyridoxal [^{32}P]phosphate

When pyridoxal, [γ-^{32}P]ATP, and pyridoxine kinase were used to synthesize pyridoxal [^{32}P]phosphate, the yields were 9—20% based on the recovery of ^{32}P from the DEAE-Sephadex A-25 column. A typical elution pattern of the

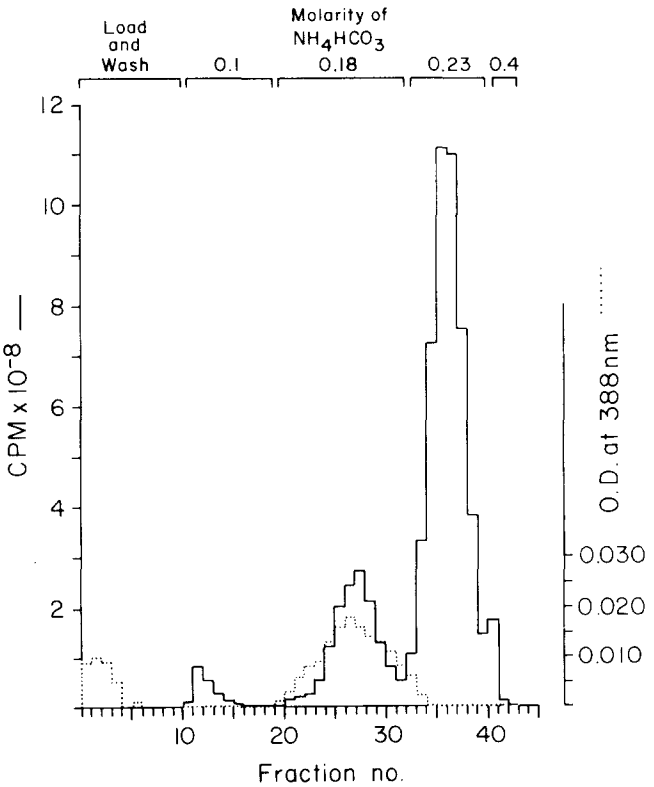

Fig. 1. DEAE-Sephadex chromatography of pyridoxal [^{32}P]phosphate. Pyridoxal [^{32}P]phosphate synthesized by pyridoxine kinase, [γ-^{32}P]ATP, and pyridoxal was fractionated in the dark on a 2—3 ml DEAE-Sephadex A-25 column after dilution of the radioactive pyridoxal-*P* 20—30-fold by carrier pyridoxal-*P*. Elution was achieved by a stepwise gradient of 0.01, 0.1, 0.18, 0.23, and 0.4 M NH_4HCO_3. Fractions of 5 ml were collected and the radioactivity (———) was monitored by counting 5-μl aliquots in Aquasol. The fractions were also monitored for adsorption by pyridoxal-*P* at 388 nm (· · · · ·). Pyridoxal absorbs only slightly at this wavelength and was eluted in the first four fractions.

reaction mixture from the DEAE-Sephadex column is shown in Fig. 1. A small amount of the carrier pyridoxal-*P* washed through the column and eluted with the load and wash. Unadsorbed pyridoxal eluted in fractions 1—5. All of the radioactivity adsorbed to the column and eluted with 0.18 M NH_4HCO_3 coincident with the marker pyridoxal-*P*. When these fractions were checked for purity by thin-layer chromatography, it was evident that they consisted of pure pyridoxal phosphate. The pyridoxal [^{32}P]phosphate produced by this procedure had a specific activity of at least 1 Ci/mmol after dilution of the reaction product with carrier pyridoxal-*P*. It was found by thin-layer chromatography that radioactive degradation products of the pyridoxal [^{32}P]phosphate began to appear after 2 days of storage of the pyridoxal [^{32}P]phosphate at −80° C. However, these products did not appear to interfere with the labeling reaction (v. infra).

Labeling of influenza virus

For the initial labeling experiments with pyridoxal [^{32}P]phosphate, the myxovirus influenza was used. This virus consists of a nucleoprotein core surrounded by a lipid envelope. When virus is grown in the absence of serum, four major proteins can be detected by ^{14}C-labeled amino acid incorporation (Fig. 2a) [7]. These are HA, the viral hemagglutinin, N, the nucelocapsid protein, NA, the viral neuraminidase, and M, the membrane protein. HA and NA have been shown by several techniques to be located on the external side of the lipid envelope and are glycoproteins, whereas N and M are located on the internal side of the viral envelope [7,28,27]. The high molecular weight species at the top of the gel (Fig. 2a) were not consistently found and may represent the incomplete dissociation of viral protein aggregates. Also, the proteins located between NA and M varied in amount and may be the result of the proteolysis of HA.

When the influenza virus grown in the absence of serum was labeled with pyridoxal [^{32}P]phosphate, repurified, and the labeled viral proteins examined by autoradiography after SDS polyacrylamide gel electrophoresis, the pattern seen in Fig. 2b was obtained. Most of the ^{32}P was associated with the glycoprotein HA and a smaller amount with NA which was incompletely resolved in the gel illustrated. The M and N proteins located on the internal side of the virion have almost no ^{32}P associated with them. The small amount of ^{32}P that appears to be associated with the M protein may in fact be associated with the protein HA_2, a degradation product of HA known to migrate only a little more slowly than the M protein. The two high molecular weight proteins were also labeled with pyridoxal [^{32}P]phosphate. Thus, the labeling pattern of the proteins of influenza virus with pyridoxal [^{32}P]phosphate is consistent with the proposition that this reagent labels only those proteins on the external surface of the viral membrane.

Labeling of erythrocytes and ghosts

A second vesiculated structure whose protein composition and disposition are known is the human erythrocyte [30]. In Fig. 3A is shown a typical slab gel pattern of the proteins of the human erythrocyte labeled with pyridoxal [^{32}P]-phosphate as visualized by Coomassie Brilliant Blue staining. When this gel was

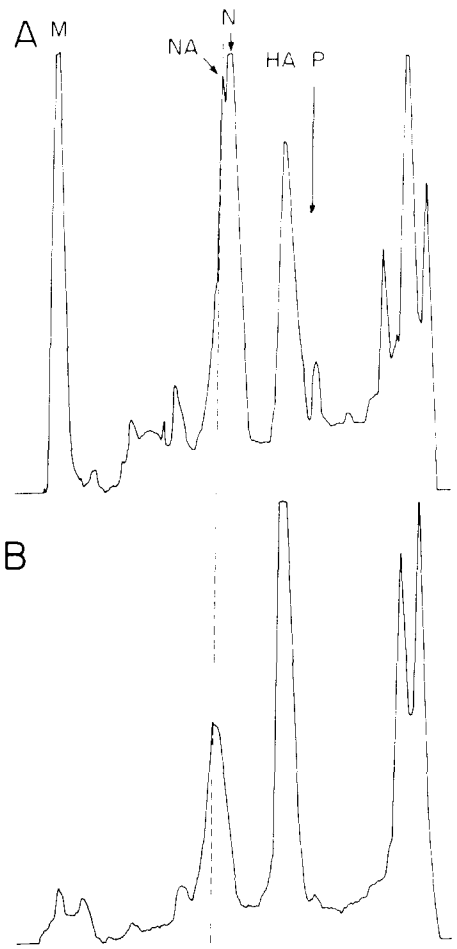

Fig. 2. Densitometer scans of autoradiographs of influenza virus proteins. Each of two samples of influenza were treated with β-mercaptoethanol and electrophoresed at 10 mA on a 10% SDS polyacrylamide slab gel. The electrophoresis was terminated 2 h after the dye front had reached the bottom of the gel. Autoradiographs were made from the dried gels and scanned on a Joyce-Loebl densitometer. The direction of electrophoresis was from right to left. All virus samples were grown in serum-free medium. HA, hemagglutinin; N, nucelocapsid; NA, neuraminidase; M, matrix protein; P, viral polymerase. (A) Virus grown in the presence of ^{14}C-labeled amino acids. (B) Virus grown in the absence of radioactive precursors and then labeled with pyridoxal [^{32}P]phosphate. The ^{32}P label is most prominent in the two external proteins HA and NA.

dried and analyzed by autoradiography for ^{32}P incorporation, the patterns in Figs. 3E and 3F were seen. Most of the radioactivity incorporated into intact erythrocytes was found in a protein band migrating with a molecular weight of approx. 100000. A small amount of radioactivity was also found at a higher molecular weight corresponding to the position of spectrin. In addition, a protein migrating near the gel front was sometimes seen to be labeled with ^{32}P. However, if the erythrocyte membranes were rigorously washed free of hemoglobin, this last radioactive peak was not found. If the erythrocytes were lysed, and isolated ghosts were labeled with pyridoxal [^{32}P]phosphate (Figs. 3C and

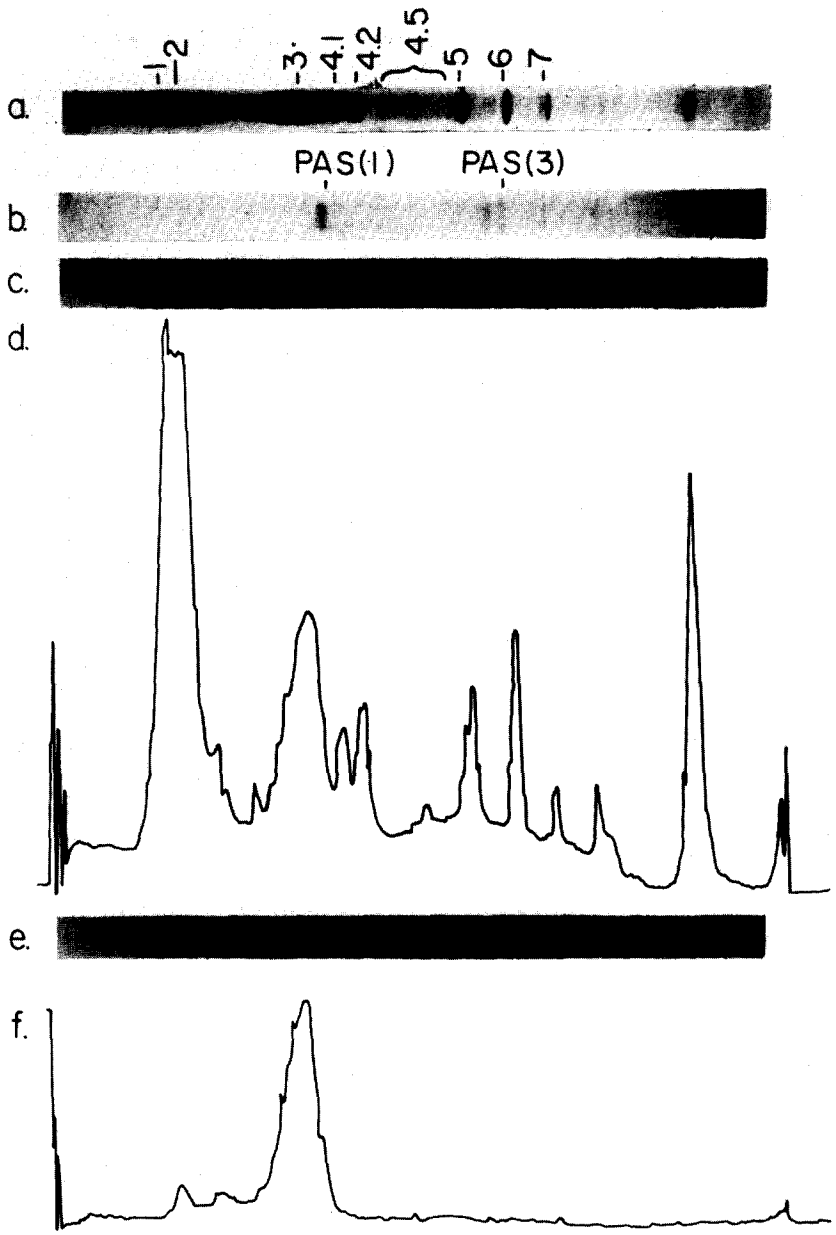

Fig. 3. Pyridoxal [^{32}P]phosphate labeling of the proteins from erythrocytes and erythrocyte ghosts. Erythrocytes and erythrocyte ghosts were labeled with pyridoxal [^{32}P]phosphate and electrophoresed on 6—16% linear gradients of polyacrylamide in the presence of SDS. 40 μg of protein were applied to each well and electrophoresis was performed at 10 mA until the dye front reached the bottom of the gel. (A) Coomassie Brilliant Blue stain of a gel of erythrocyte ghosts which had been labeled with pyridoxal [^{32}P]phosphate. (B) PAS stain of gel of erythrocyte ghost proteins. (C) Autoradiograph of gel A indicating which proteins had been labeled with pyridoxal [^{32}P]phosphate. (D) Densitometer tracing of autoradiograph illustrated in C. (E) Autoradiograph of a gel of pyridoxal [^{32}P]phosphate-labeled intact erythrocytes. (F) Densitometer tracing of autoradiograph illustrated in part E. Major proteins and glycoproteins are numbered according to Steck [30]. The direction of electrophoresis was from left to right in all samples.

3D) essentially all of the proteins were labeled. Under these circumstances, a peak of radioactivity corresponding to hemoglobin was almost always found.

Other studies employing membrane-impermeable reagents to label the external proteins of erythrocytes have shown that the protein species predominantly labeled are component 3 and the glycoprotein, PAS(1) [30]. To determine if PAS(1) was labeled, slab gels of pyridoxal [^{32}P]phosphate-labeled erythrocytes were stained for carbohydrate and analyzed for radioactivity. At least five PAS-positive proteins, corresponding to the major glycoproteins, could be detected by this method (Fig. 3B). When the gel was analyzed for ^{32}P bound to whole erythrocytes, the major radioactive peak did not correspond to PAS(1) (Figs. 3B and 3E). The glycoprotein had a slightly faster mobility than the radioactive species. Moreover, when ghosts were labeled and the radioactivity profile (Fig. 3C) was compared to the glycoprotein profile, it was obvious that even under these conditions the major glycoprotein was not labeled. With this method, then, the major protein species which is labeled in intact erythrocytes is component 3 and not glycoprotein PAS(1).

The small amount of radioactivity seen with the hemoglobin and spectrin peaks (Fig. 3F) indicated that pyridoxal [^{32}P]phosphate might be penetrating the red cell membrane and complexing with these two proteins. Although the amount of radioactivity associated with the hemoglobin peak was relatively low, because of the large amount of hemoglobin per erythrocyte, the total amount of ^{32}P incorporated into hemoglobin was seven times that found associated with the external proteins. Since it is known that hemoglobin has an extremely high affinity for pyridoxal-*P* [31], a small number of lysed red cells may have contributed protein that might bind to the red blood cell membrane, label with pyridoxal [^{32}P]phosphate, and contaminate the final membrane preparation. Pyridoxal [^{32}P]phosphate-hemoglobin was prepared to test this possibility and a reconstruction experiment performed. When erythrocytes or ghosts were mixed with pyridoxal [^{32}P]phosphate-hemoglobin, reisolated, and their proteins analyzed for ^{32}P after SDS polyacrylamide gel electrophoresis, no radioactive hemoglobin was found associated with the preparation (results not shown). Thus, the pyridoxal [^{32}P]phosphate-hemoglobin detected in earlier reactions must have been the result of the pyridoxal [^{32}P]phosphate passing through the membrane and complexing with the hemoglobin.

Labeling of normal and transformed chicken embryo fibroblasts

A number of workers have described a difference in the external membrane proteins of normal and transformed chicken cells as visualized on polyacrylamide gels [32—35]. The normal cells contain a glycoprotein of approx. 220000 molecular weight located on the external surface of the plasma membrane. Transformed cells appear to be missing this protein, as it is not seen either by labeling cells with membrane-impermeable agents such as lactoperoxidase, ^{125}I and glucose oxidase and NaB^3H_4 or by Coomassie Blue staining of total cellular proteins. As illustrated in Fig. 4, when the proteins of normal and transformed chicken cells are examined with pyridoxal [^{32}P]phosphate labeling followed by polyacrylamide gel electrophoresis, normal cells are seen to contain a high molecular weight ^{32}P-labeled protein missing in Rous sarcoma virus-transformed cells.

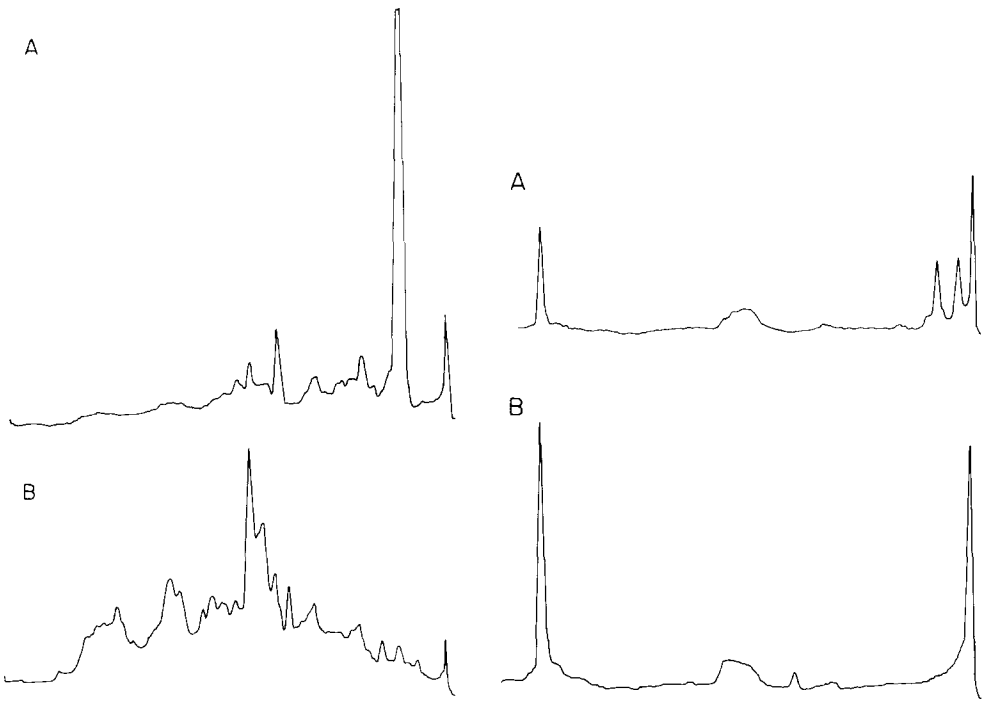

Fig. 4. Pyridoxal [^{32}P]phosphate labeling of proteins of normal and Rous sarcoma virus-transformed chick embryo fibroblasts. Normal and Rous sarcoma virus-transformed chick fibroblasts were labeled with pyridoxal [^{32}P]phosphate as described in the text. 40 μg of each sample were then applied to a 6—16% linear SDS polyacrylamide gradient slab gel and electrophoresed at 10 mA until the dye front reached the bottom of the gel. The gels were then dried, autoradiographs made and scanned with a Joyce-Loebl densitometer. (A) Densitometer scan of an autoradiograph of normal chick cell proteins labeled with pyridoxal [^{32}P]phosphate. (B) Densitometer scan of Rous sarcoma virus-transformed chick cells labeled with pyridoxal [^{32}P]phosphate. The direction of electrophoresis was from right to left.

Fig. 5. Pyridoxal [^{32}P]phosphate labeling of proteins from normal and Rous sarcoma virus-transformed chick embyro fibroblasts. The conditions for labeling and electrophoresis were identical to those described in Fig. 4 except that the polyacrylamide gel was 8%. (A) Densitometer scan of an autoradiograph of normal chick cell proteins labeled with pyridoxal [^{32}P]phosphate. (B) Densitometer scan of Rous sarcoma virus-transformed chick cells labeled with pyridoxal [^{32}P]phosphate. The direction of electrophoresis was from right to left.

If the proteins from normal and transformed cells were analyzed on an 8% (Fig. 5) gel, rather than on a 6—16% (Fig. 4) gel, the single protein containing ^{32}P could be resolved into two species, both of which were absent in the transformed cell preparation. The absence of these two proteins in transformed chick cells was described earlier by Wickus et al. [36].

Since the majority of the proteins in either the normal or the transformed cells do not incorporate pyridoxal [^{32}P]phosphate, it appears that this reagent can be used successfully with living cells to label external surface proteins. Further proof of the external position of the ^{32}P-containing proteins comes from the demonstration that all of the radioactive label associated with the cells can be removed by mild protease treatment (trypsin 5 μg/ml, 10 min, 37° C) of intact cells.

Discussion

These experiments demonstrate that pyridoxal phosphate labeled with ^{32}P can be produced in sufficient yield to be employed as a reagent for identifying proteins on the surfaces of membranes. The enzyme used to catalyze the synthesis, pyridoxine kinase, can be prepared with a minimum of effort and is extremely stable once isolated. The preparation used in these experiments was not homogeneous, as several protein bands could be seen after SDS polyacrylamide gel electrophoresis. The enzyme preparation, however, was judged to be free of contaminating phosphatases and was suitable for the production of pyridoxal [^{32}P]phosphate. Since absolute purity of the enzyme is not required, enzyme preparations at earlier steps during the purification might be successfully employed for pyridoxal-*P* synthesis provided the phosphatases are absent.

The labeling reactions of pyridoxal [^{32}P]phosphate with influenza virus and normal and transformed chicken fibroblasts yielded patterns essentially equivalent to those seen earlier using either pyridoxal-*P* and NaB^3H_4 or other reagents. Thus this reaction appears to be limited to proteins on the external surface of the viral or plasma membrane.

The labeling of human erythrocytes was more complex than that of either influenza virus or chicken fibroblasts. Some reagent did appear to pass through the cell membrane and reacted with two intracellular proteins, hemoglobin and spectrin. The ability of pyridoxal-*P* to pass through the red blood cell membrane has been reported previously [37], and is thought to be mediated by the anion transport system known to be present in these cells. The major external membrane protein that was labeled was a non-glycosylated protein with a molecular weight of approx. 100000. The failure to label the major external glycoprotein, PAS(1), was surprising in view of the report of Cabantchik et al. [37] describing the labeling of this protein by pyridoxal-*P*. The difference between our results and those of Cabantchik et al. [37] may be related to the low concentrations of pyridoxal-*P* only the 100000 molecular weight protein is labeled while at higher concentrations of pyridoxal-*P*, the glycoproteins are labeled. However, the concentration of labeling reagent used did not seem to be limiting since the same concentration of pyridoxal-*P* labeled most of the other erythrocyte proteins in ghost preparations. It should also be noted that Cabantchik et al. [37] used a pH of 6.6 for their experiments whereas we used a pH of 7.4. The lack of PAS(1) reactivity with our reagent may also be the result of a repulsion between the negatively charged sialic acid residues of the protein and the phosphate group in the pyridoxal-*P*. Since the reactive moiety in pyridoxal-*P* is located close to the phosphate group, this may preclude its reacting with proteins that contain highly negatively charged sugar residues. These aspects of the labeling reaction can be tested in future experiments.

Acknowledgements

The authors wish to thank Kimberly Waterman for his valuable contribution and Drs. S.T. Rohrlich, W. Beers, and E. Reich for their comments. We are grateful to Dr. W. Dempsey for giving us unpublished information concerning the purification of pyridoxine kinase. This research was supported by Grant

Ca13138 from the National Institutes of Health. D.B.R. holds a Faculty Research Grant from the American Cancer Society.

References

1 Berg, H.C. (1969) Biochim. Biophys. Acta 183, 65—78
2 Phillips, D.R. and Morrison, M. (1970) Biochem. Biophys. Res. Commun. 40, 284—289
3 Glaser, M., Simpkins, H., Singer, S.J., Sheetz, M. and Chan, S.I. (1970) Proc. Natl. Acad. Sci. U.S. 65, 721—728
4 Bretscher, M.S. (1971) J. Mol. Biol. 58, 775—781
5 Carraway, K.L. and Shin, B.C. (1972) J. Biol. Chem. 247, 2102—2108
6 Hubbard, A.I. and Cohn, Z.A. (1972) J. Cell Biol. 55, 390—405
7 Rifkin, D.B., Compans, R.W. and Reich, E. (1972) J. Biol. Chem. 247, 6432—6437
8 Karlin, A. (1973) Fed. Proc. 32, 1847—1853
9 Ruoho, H.E., Hansruedi, K., Roeder, P.E. and Singer, S.J. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2567—2571
10 Steck, T.L. and Dawson, G. (1974) J. Biol. Chem. 249, 2135—2142
11 Jackson, R.C. (1975) J. Biol. Chem. 250, 617—622
12 Starov, J.V. and Richards, F.M. (1975) J. Biol. Chem. 250, 8174—8178
13 Heitzmann, H. and Richards, F.M. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3537—3541
14 Whiteley, N.M. and Berg, H.C. (1974) J. Mol. Biol. 87, 541—561
15 White, R.S. and Dempsey, W. (1970) Biochemistry 9, 4057—4060
16 White, R.S. and Dempsey (1970) in Methods in Enzymology (McCormick, D.B. and Wright, L.D., eds.), Vol. XVIII, pp. 619—622, Academic Press, New York
17 Garen, A. and Leventhal, C. (1960) Biochim. Biophys. Acta 38, 470—481
18 Schendel, P.F. and Wells, R.D. (1973) J. Biol. Chem. 248, 8319—8321
19 Randerath, K. and Randerath, E. (1966) in Methods in Enzymology (McCormick, D.B., ed.), vol. XII, pp. 322—347, Academic Press, New York
20 Ahrens, H. and Korytnyk, M. (1969) Anal. Biochem. 30, 415—420
21 Fairbanks, G., Steck, T.L. and Wallack, D.F.H. (1971) Biochemistry 10, 2607—2617
22 Rifkin, D.B. and Reich, E. (1970) Virology 45, 172—181
23 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119—130
24 Korn, E.D. and Wright, P.C. (1973) J. Biol. Chem. 248, 439—447
25 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—273
26 Laemmli, U.K. (1970) Nature 227, 680—684
27 Studier, W. (1973) J. Mol. Biol. 79, 237—248
28 Compans, R.W., Klenk, H.-D., Caliguiri, L.A. and Choppin, P.W. (1970) Virology 22, 880—889
29 Schulze, I.T. (1970) Virology 42, 890—904
30 Steck, T. (1974) J. Cell Biol. 62, 1—19
31 Benesch, R., Ung, S., Suzuki, T., Bauer, C. and Benesch, R. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2595—2599
32 Bussell, R.H. and Robinson, W. (1973) J. Virol. 12, 320—327
33 Wickus, G.G. and Robbins, P.W. (1973) Nat. New Biol. 245, 65—67
34 Marciani, D.J. and Bader, J.P. (1975) Biochim. Biophys. Acta 401, 386—398
35 Stone, K.R., Smith, R.E. and Joklik, W.K. (1974) Virology 58, 86—100
36 Wickus, G.G., Brnaton, P.E. and Robbins, P.W. (1974) in Control of Proliferation in Animal Cells (Clarkson, B. and Baserga, R., eds.), pp. 541—546, Cold Spring Harbor Press, Cold Spring Harbor
37 Cabantchik, I.Z., Bolshin, M., Breuer, W. and Rothstein, A. (1975) J. Biol. Chem. 25, 5130—5136