SUMMARY

We describe a convenient, rapid, and reproducible method for labeling proteins in vitro by acetylation with [3H] or [14C]acetic anhydride dissolved in small amounts of anhydrous dioxane. The reaction is carried out at neutral pH and does not require the use of detergents, water-immiscible organic solvents, oxidizing, or reducing agents. Thus undesirable solvent-induced alterations in protein structure and biological activity are minimized.

A method for calculating the specific activity of the protein and the efficiency of acetylation at known concentrations of protein and acetic anhydride is presented.

Radioacetylated proteins were shown to be suitable for use as molecular weight calibration standards and as protein markers in polyacrylamide gel electrophoresis, gel filtration, and enzyme studies. Acetic anhydride was used to label intact oncornaviruses, which consist of a complex ribonucleoprotein core within a lipid envelope. Some of the viral lipid and all of the viral proteins, including the internal ones, were labeled without detectable alterations in viral morphology or buoyant density. This result suggests that acetic anhydride, evidently by virtue of its small size and neutral charge, penetrates freely throughout the viral membrane and core structures.

The reactivity of RNA with acetic anhydride was less than 1% that of protein under similar reaction conditions.

Although a large number of procedures for labeling proteins with a variety of radioisotopes have been described, each has been of limited applicability. Among the most commonly employed procedures are iodination, methylation, and formaldehyde-borohydride reduction. Iodination of proteins by the chloramine-T method (6) may be used to radioiodinate proteins to a high specific activity, but the relatively short half-life of the iodine radionuclides is a distinct disadvantage. In addition, this method appears to be unsuitable for proteins lacking tyrosine, e.g. secretin and porcine parathyroid hormone, and for a number of proteins which lose antigenicity or enzymatic activity under the harsh reaction conditions required for iodination (7).

Enzymatic radioiodination of proteins with lactoperoxidase (8-10) eliminates the exposure of the protein to strong oxidizing (chloramine-T) and reducing (sodium metabisulfite) agents, but evidence has been presented suggesting that some proteins may be damaged by exposure to components present in commercial [125I] solutions (7).

Radioactivity methods employing 3H and 14C radionuclides also have been described. An early procedure, adapted from RNA radioiodination (11), was to methylate proteins with radioactive dimethylsulphate dissolved in benzene (12). However the complications associated with the two-phase reaction mixture and the denaturing effect of benzene on proteins and membranes restricts the applicability of this method. More recently a method of radioiodinating proteins by reductive methylation with radioactive formaldehyde in conjunction with sodium borohydride has been reported (13). Again the versatility of this method is limited by the necessity of carrying out the reaction at pH 9 and 0-2, and by the possible harmful effects of the strong reducing agent on protein structure and biological activity.

We report here conditions for radioiodinating proteins and intact viruses by acetylation with [3H]- or [14C]acetic anhydride dissolved in small quantities of anhydrous dioxane. Acetic anhydride was chosen as a labeling agent because: (a) its small size minimizes the effect of substitution on the molecular weight of the protein, (b) it reacts at neutral pH with a variety of functional groups, and (c) it is available commercially at lower cost and higher specific activity than the corresponding radioactive dimethylsulphate or formaldehyde. The use of water-miscible dioxane as an inert solvent for handling minute quantities of carrier-free acetic anhydride enables one to radioiodinate proteins in a single phase reaction mixture which is gentle to proteins and membranes.
MATERIALS AND METHODS

Proteins
Lysozyme, lima bean trypsin inhibitor, carboxypeptidase A, phosphorylase a, and catalase were obtained from Worthington; bovine serum albumin, ovalbumin, and chymotrypsinogen A from Schwarz-Mann; 7 S γ-globulin, apoferritin, ribonuclease A, cytochrome c, and myoglobin from Sigma; and zinc insulin from United States Reference Standards.

Preparation of Proteins for Labeling
Proteins, except as noted below, were dissolved in 0.3 M sodium phosphate, pH 7.2, to a concentration of 10 mg per ml. Phosphorylase a, carboxypeptidase A, and catalase were purchased and repurified as suspensions containing 4.6, 10, and 3.25 mg per ml, respectively. Protein samples containing compounds reactive with acetic anhydride, e.g., Tris buffers, 2-mercaptoethanol, or ammonium salts, were dialyzed before acetylation against 0.3 M sodium phosphate, pH 7.2, at 5°. Insulin was acetylated either as the insoluble zinc complex or in the soluble form produced after removal of zinc by dialysis against the phosphate buffer.

Anhydrous Dioxane
Anhydrous dioxane was prepared by refluxing reagent grade dioxane over sodium metal (about 1 g/100 ml of dioxane) for 3 hours under a slow nitrogen stream. The dioxane then was distilled from the sodium metal taking care not to distill to dryness. This distilled dioxane again was refluxed over sodium for 1 hour and redistilled to remove the reagent. The molten sodium metal beads should remain bright and silvery indicating the refluxing dioxane is indeed anhydrous. The anhydrous dioxane was stored under dry nitrogen in sealed 5-ml glass ampules (Kimble Catalog No. 12012-L). Some workers may find lithium aluminum hydride a more convenient reagent for drying dioxane (11).

Preparation and Storage of Acetic Anhydride
To ensure recovery and prevent escape of the minute volumes (10 to 50 μl) of radioactive acetic anhydride (boiling point, 140°) which are purchased commercially in evacuated breakseal ampules, the reagent was concentrated on the bottom of the ampule by placing the lower end of the container in Dry Ice under an infrared lamp for 30 to 45 min. The acetic anhydride “distills” to the bottom of the ampule where it is frozen and concentrated. In cases where the entire sample of acetic anhydride was to be used for a single acetylation, the solution of protein to be labeled was added directly to the freshly opened vial containing the “concentrated” reagent. In those cases where the anhydride was to be used for acetylation of more than one protein, the reagent was dissolved in anhydrous dioxane and divided into the appropriate aliquots.

Several methods for storing the reagent were used.
Procedure A—[H]Acetic anhydride, 25 mCi (New England Nuclear; specific activity, 50 mCi/mmole), was dissolved in 2 ml of anhydrous dioxane to give a final concentration of 250 μmol per ml. One-milliliter portions were stored in the refrigerator in conical glass screw-cap containers (Reacti-Vial, 1-ml capacity; Pierce Chemical Co., Rockford, III.) and sealed with waterproof tape to exclude atmospheric moisture. Under these conditions the reagent retained its reactivity for a period of several months.
Procedure B—[14C]Acetic anhydride, 2.5 mCi (Dohm Co., Hollywood, Calif.; specific activity, 30 mCi per mmole), 33 μmol per ml in anhydrous dioxane, was prepared as described in Procedure A except that the taped screw containers were stored at −70°. This procedure improved, but did not solve, the hydrolysis problem because it was difficult to exclude atmospheric water when the vial was opened to remove the reagent.
Procedure C—Five milliequivalents of [H]acetic anhydride (New England Nuclear; specific activity, 50 mCi per mmole) was dissolved in 0.2 ml of anhydrous dioxane. Ten-microliter aliquots, representing 5 μmol of acetic anhydride, were sealed under dry nitrogen in 1-ml glass ampules and stored frozen at −70°. Stored in this manner the reagent exhibited no significant decline in reactivity even after 18 months. Each vial was used for one acetylation, thereby eliminating the introduction of moisture into the stock anhydride.

Acetylation Procedure
In a typical experiment 1 ml of 1% (w/v) protein in 0.3 M sodium phosphate, pH 7.2, was mixed with radioactive acetic anhydride in dioxane and incubated for 30 min at room temperature, when virtually all of the anhydride was reacted either with water or protein. If desired the reaction may be stopped early by hydrolyzing the residual anhydride with a 10-fold excess of 2-mercaptoethanol. Pure 2-mercaptoethanol is 14.3 M. The radioactive proteins then were separated from the acetic anhydride hydrolysis products by gel filtration on a column (1.4 X 24 cm) of Sephadex G-25 fine (Pharmacia, Uppsala, Sweden), equilibrated, and eluted with 0.01 M sodium phosphate, pH 7.2 (e.g., Fig. 1). Dialysis is a simple alternative, but the risk of protein degradation by trace amounts of proteases, either endogenous or introduced during handling, is increased because of the longer purification time required.

The amount of radioactive acetic anhydride used in each experiment depends on the degree of acetylation required (see, for example, Fig. 2). As a rule of thumb, for protein concentrations less than 1%, 5 μmol of acetic anhydride per ml of reaction mixture typically yields an acetylation density of 1 acetyl residue per 10,000 daltons of protein.

 Determination of Radioactivity
Procedure 1—Samples, diluted to 0.5 ml in 0.02 M sodium phosphate, pH 7.2, containing 0.14 M sodium chloride, were counted in 10 ml of scintillation solvent B-10. Scintillation solvent B-10 consists of 10% (w/v) Beckman solvent BBS-3, 0.5% 2,5-di-phenyloxazole (POP) and 0.01% 1,4-bis-(2-(4-methyl-5-phenyl-oxazoyl)phenyl)benzene (dimethyl POPPOP) in toluene.
Procedure 2—Samples, up to 0.1 ml, were transferred to filter paper discs (Whatman No. 3MM, 2.3 cm) which were sequentially washed in 5% (w/v) trichloroacetic acid, ethanol diethyl ether (1:1), and finally diethyl ether. Each treatment was for 15 min in a minimum of 20 ml of solvent per disc. After air drying, the discs were counted in 10 ml of scintillation solvent PPOT. PPOT

Fig. 1. Gel filtration of [14C]-radioacetylated bovine serum albumin (BSA). A 1.0 ml reaction mixture containing 1% albumin and 1.9 μmol of [14C]acetic anhydride ([14C]Ac) in 50 μl of anhydrous dioxane (Stock B, “Materials and Methods”) was desalted on a column (1.4 X 20 cm) of Sephadex G-25 (fine) eluted with 0.01 M sodium phosphate, pH 7.2, at a flow rate of about 12 ml per hour. Samples (5 μl) of each fraction (0.5 ml) were assayed for radioactivity in B-10 scintillation solvent. The shoulder preceding the [14C]acetate peak was present in all of the acetylated preparations. No attempt was made to identify it.
about 15,000 dpm each of the acetylated marker proteins bovine serum albumin, chymotrypsinogen \(\alpha\), lysozyme, and insulin in a total volume of 15 \(\mu\)l were added to 200 \(\mu\)l of 6 M Gdn-HCl (Schwarz-Mann, Orangeburg, N. Y.) in 0.5 M Tri, pH 8.6, containing 0.5% 2-mercaptoethanol and heated at 45° for 1 h. The reduced proteins then were carboxymethylated by adding 35 \(\mu\)l of iodoacetamide and heating in the dark at 45° for 30 min. To stop the reaction excess iodoacetamide was destroyed by adding 10 \(\mu\)l of 2-mercaptoethanol. Blue dextran (5 \(\mu\)l of a 2% solution in 6 M Gdn-HCl) and dinitrophenyl alanine (1 \(\mu\)l of a 2.5% solution in 0.1 N NaOH) were added as visual markers and 20 \(\mu\)g of sucrose were added to facilitate sample layering.

Gel filtration was carried out on a column (0.85 X 71 cm) consisting of Bio-Rad Agarose A-5m (Bio-Rad Laboratories, Richmond, Calif.) equilibrated and eluted with 6 M guanidine hydrochloride, pH 7.2. Fractions (0.5 ml) were collected directly into scintillation vials at a flow rate of 1.8 ml per hr. Each fraction was dissolved in 5 ml of Aquasol scintillation solvent (New England Nuclear) and counted in a Packard Tri-Carb spectrometer at 6" or in a Beckman scintillation counter LS-233 at 25°. Absorbance was read at 630 nm for blue dextran and at 390 nm for sodium DNP-alanine. The standard curves were constructed according to the procedure of Fish et al. (5).

Radioacetylation of Avian Myeloblastosis Virus

Radioacetylation of Intact AMV—A 500-\(\mu\)l sample of purified AMV (about 0.8 mg per ml) in 0.2 M sodium phosphate, pH 7.2, containing about 30% sucrose was added to a freshly opened ampule containing 5 \(\mu\)mol of \(^{14}C\)acetate in 10 \(\mu\)l of dimethyl sulfoxide (see "Materials and Methods," Stock C). After 30 min at room temperature, the virus was purified from the unreacted \(^{14}C\)acetate by desalting the reaction mixture through a Sephadex G-25 column (1.4 X 25 cm) eluted with 0.01 M sodium phosphate, pH 7.2. A portion (0.4 ml) of the radioacetylated AMV then was diluted to 5.2 ml with 0.01 M sodium phosphate, pH 7.2, containing 0.1% bovine serum albumin and 1 mM EDTA, and the virus was pelleted in a SW 65 rotor by centrifuging at 45,000 rpm for 45 min at 6°. The virus pellet was redisolved in 100 \(\mu\)l of 0.01 M sodium phosphate, pH 7.2, containing 1% sodium dodecyl sulfate and 2-mercaptoethanol by heating in a boiling water bath for 2 min. After adding sucrose crystals to a concentration of 10%, the virus sample was subjected to electrophoresis on a 25-cm gel of 10% acrylamide for 19 hours at 8 ma per gel. The gel then was sliced automatically into 1-mm fractions and each fraction was analyzed in scintillation solvent T21 for radioactivity as described previously.

Radioacetylation of Sodium Dodecyl Sulfate-disrupted AMV—A 500-\(\mu\)l sample of the same AMV preparation was disrupted prior to acetylation by adding 10% sodium dodecyl sulfate to a final concentration of 1% and heating the virus in a boiling water bath for 2 min. The disrupted virus then was cooled and added to 5 \(\mu\)mol of \(^{14}C\)acetate in 10 \(\mu\)l of dimethyl sulfoxide in a boiling water bath at 6°. After adding sucrose crystals to a concentration of 10%, the virus sample was subjected to electrophoresis on a 25-cm gel of 10% acrylamide for 19 hours at 8 ma per gel. The gel then was sliced automatically into 1-mm fractions and each fraction was analyzed in scintillation solvent T21 for radioactivity as described previously.

RESULTS

Kinetics, Density, and Efficiency of Acetylation—The kinetics of acetylation of bovine serum albumin at 25° under typical reaction conditions was determined by a filter paper assay as described in Fig. 3. The reaction of acetic anhydride with protein was very rapid; acetylation was about 70% complete

1 The abbreviations used are: Gdn-HCl, guanidine hydrochloride; AMV, avian myeloblastosis virus.
The reaction period could be reduced, if desired, to 5 to 10 min with decreasing the bovine serum albumin concentration from 10 to 0.1 mg per ml at a constant concentration of acetic anhydride by water. The half-life of acetic anhydride in 0.05 mM KCl at pH 7.2 was about 3 min at 25°C. Using this value, the concentration of acetic anhydride remaining after 30 min (10 half-lives) would be less than 0.1% of the initial value. In view of these considerations a 30-min reaction period was adopted for routine acetylations. The kinetics suggest, however, that the reaction period could be reduced, if desired, to 5 to 10 min with only a 10 to 15% reduction in the specific activity of the labeled protein. A similar, but somewhat slower, rate of reaction was observed when acetylations were carried out at 4°C (data not shown).

To determine the effect of acetic anhydride concentration on the number of acetyl residues incorporated per unit mass of protein (the acetylation density), a constant amount of bovine serum albumin was treated with increasing concentrations of acetic anhydride (Fig. 2). Under these reaction conditions, the acetylation density was directly proportional to acetic anhydride concentrations up to at least 40 μM (Column 2). Observed densities were calculated as described under "Materials and Methods." The theoretical values are calculated from Equation 7 (see text).

Table I

<table>
<thead>
<tr>
<th>Concentration of bovine serum albumin mg/ml</th>
<th>Millions of H+ dpm incorporated</th>
<th>Millions of H+ dpm per mg protein*</th>
<th>Acetylation efficiency*</th>
<th>Acetylation density*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>19</td>
<td>0.036</td>
<td>0.35</td>
<td>0.35</td>
</tr>
<tr>
<td>0.2</td>
<td>20</td>
<td>0.072</td>
<td>0.36</td>
<td>0.36</td>
</tr>
<tr>
<td>0.4</td>
<td>19</td>
<td>0.014</td>
<td>0.35</td>
<td>0.35</td>
</tr>
<tr>
<td>1.0</td>
<td>18</td>
<td>0.032</td>
<td>0.32</td>
<td>0.32</td>
</tr>
<tr>
<td>4.0</td>
<td>16</td>
<td>0.113</td>
<td>0.29</td>
<td>0.29</td>
</tr>
<tr>
<td>10</td>
<td>14</td>
<td>0.255</td>
<td>0.26</td>
<td>0.26</td>
</tr>
</tbody>
</table>

The effect of protein concentration on the acetylation density and the efficiency of reagent utilization achieved with a constant concentration of acetic anhydride is summarized in Table I. Decreasing the bovine serum albumin concentration from 10 to 0.1 mg per ml at a constant concentration of acetic anhydride (10 μM per ml) decreased the efficiency of anhydride utilization after 3 min and virtually 100% complete by 25 min. Since only about three of the 80 to 90 potentially reactive sites per molecule (18) were acetylated, cessation of the reaction was evidently not due to saturation of reactive sites, but rather to hydrolysis of anhydride by water. The half-life of acetic anhydride in 0.05 mM KCl at pH 7.2 was about 3 min at 25°C. Using this value, the concentration of acetic anhydride remaining after 30 min (10 half-lives) would be less than 0.1% of the initial value. In view of these considerations a 30-min reaction period was adopted for routine acetylations. The kinetics suggest, however, that the reaction period could be reduced, if desired, to 5 to 10 min with only a 10 to 15% reduction in the specific activity of the labeled protein. A similar, but somewhat slower, rate of reaction was observed when acetylations were carried out at 4°C (data not shown).

The effect of protein concentration on the acetylation density and the efficiency of reagent utilization achieved with a constant concentration of acetic anhydride is summarized in Table I. Decreasing the bovine serum albumin concentration from 10 to 0.1 mg per ml at a constant concentration of acetic anhydride (10 μM per ml) decreased the efficiency of anhydride utilization from 26 to 0.4% (Column 4). This decrease was accompanied by a slight increase in protein specific activity, a measure of acetylation density, from 14 × 10^6 dpm per mg of protein to a maximum of about 19 × 10^6 dpm per mg at a protein concentration of 1 mg per ml (Column 3). Below this concentration of protein, the specific activity remained constant at about 18 to 20 × 10^6 dpm per mg.

**Prediction of Acetylation Efficiencies and Densities—**At neutral pH acetic anhydride reacts with the amino, phenolic, sulfhydryl, and imidazol groups of the protein side chains. Since the products of the latter two are relatively unstable and hydrolyze at neutral pH, the net acetylation is predominantly of the lysyl and tyrosyl residues (Equations 2a and 2b) (19). The competing hydrolysis reaction is summarized in Equation 3.

\[ \text{Ac\textsuperscript{14}O + Pr - NH}_3^+ \overset{K'}{\underset{\text{pH 7.2}}{\rightleftharpoons}} \text{Pr - NH - Ac} + \text{AcO}^- + 2 \text{H}^+ \]  
(2a)

\[ \text{Ac\textsuperscript{14}O + Pr - Tyr - OH} \overset{K''}{\underset{\text{pH 7.2}}{\rightleftharpoons}} \text{Pr - Tyr - OAc} + \text{AcO}^- + 2 \text{H}^+ \]  
(2b)

\[ \text{Ac\textsuperscript{14}O + HOH} \overset{K_1}{\underset{\text{pH 7.2}}{\rightarrow}} 2 \text{HOAc}^+ + 2 \text{H}^+ \]  
(3)

where Pr = protein, Ac\textsuperscript{14}O = labeled acetyl group.

Unpublished observations.
An important consideration in defining reaction conditions for radiolabeling proteins is to achieve the desired acetylation density at minimum cost, i.e. to utilize the radiolabeling reagent as efficiently as possible. In theory 100% efficiency (50% incorporation of label) could be obtained using anhydrous protein. In practice this is inconvenient (20) and unnecessary since reasonably good yields can be achieved in aqueous solution. For example, efficiencies of 30 to 50% were achieved under the reaction conditions described in Table II.

Rate equations which give reasonable estimates of acetylation efficiency and acetylation density as a function of protein and acetic anhydride concentration were derived as follows. As noted above, the rate \( \frac{-dA}{dt} \) of acetic anhydride consumption is the sum of the rates at which it is hydrolyzed and at which it acetylates protein. Assuming both reactions, at constant pH, are second order processes, the rate equation is:

\[
\frac{-dA}{dt} = K[A][H_2O] + K_1[A][P]
\]  

(4)

where \([A]\), \([H_2O]\), and \([P]\) are the concentrations of acetic anhydride, water, and protein, respectively; \(K_1\) is the hydrolysis rate constant and \(K_1\) is the rate constant of acetylation of bovine serum albumin in 0.3 M sodium phosphate, pH 7.2. Actually \(K_1\) is the sum of the rate constants of the reactive side chains in the protein molecule.

By limiting the acetic anhydride concentrations in the reaction vessel, it is relatively easy to define conditions so that the concentrations of water and reactive protein are effectively constant, i.e. do not change appreciably throughout the course of the experiment. For example, only about three of the more than 80 potentially reactive sites of the bovine serum albumin molecule reacted under the conditions described in Fig. 3. Under these conditions, and provided that the reaction is conducted at constant pH, the competing acetylation and hydrolysis reactions reduce to parallel first order processes (Equations 5a and 5b),

\[
\text{(acetylation)} \ A \xrightarrow{K'} \ AP
\]

(5a)

\[
\text{(hydrolysis)} \ A \xrightarrow{K} \ A\text{-OH}
\]

(5b)

where \(K' = K_1[P]\) and Equation 4 simplifies to Equation 6

\[
\frac{-dA}{dt} = [A][K + K']
\]  

(6)

For parallel first order processes it can be shown (21) that the fraction of acetic anhydride, \(A_1\), directed into the acetylation reaction (Equation 5a) at infinite time is \(K'/K + K'\). In other words the efficiency, \(E\), with which acetic anhydride is used in the acetylation of protein is

\[
E = K'/K + K' = K_1 P/(K + K_1 P)
\]  

(7)

The acetylation density, \(D\) (in micromoles of acetyl residue per mg of protein), is simply the product of the acetylation efficiency (mole fraction of acetic anhydride successful in acetylation) and the initial concentration of acetic anhydride, \(A_1\) (micromoles per ml), divided by the concentration of protein, \(P\) (milligrams per ml), in the reaction mixture:

\[
D = A_1 K_1 P/(K + K_1 P)
\]  

(8)

Conversion of Equation 7 to the linear form (Equation 9) shows that separate evaluation of \(K\) and \(K_1\) is unnecessary;

\[
l/E = \left(\frac{K}{K_1} \cdot \frac{1}{P}\right) + 1
\]

(9)

Thus by using the data in Table I, the slope of the plot of \(1/E\) versus \(1/P\) was found to be \(K/K_1 = 0.036\). Substitution in Equations 7 and 8 yields Equations 10 and 11, which predict

\[
E = 0.036P/(1 + 0.036P)
\]

(10)

\[
D = 0.036A_1/(1 + 0.036P)
\]

(11)

the acetylation efficiency and acetylation density at any concentration of bovine serum albumin in 0.3 M sodium phosphate, pH 7.2. As shown in Table I (Columns 4 and 5) the efficiency values predicted by Equation 10 were in excellent agreement with those actually observed over a wide range of protein concentra-

Table II

Properties of 14C-acetylated proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight</th>
<th>Specific activity</th>
<th>Acetylation density</th>
<th>Acetyl residues per molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-Globulin</td>
<td>160,000</td>
<td>1.0</td>
<td>0.29</td>
<td>4.6</td>
</tr>
<tr>
<td>Phosphorylase a</td>
<td>94,000</td>
<td>0.45</td>
<td>0.14</td>
<td>1.3</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>68,000</td>
<td>1.7</td>
<td>0.50</td>
<td>3.4</td>
</tr>
<tr>
<td>Catalase</td>
<td>60,000</td>
<td>0.24</td>
<td>0.07</td>
<td>0.43</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>43,000</td>
<td>0.51</td>
<td>0.15</td>
<td>0.66</td>
</tr>
<tr>
<td>Carboxypeptidase A</td>
<td>34,000</td>
<td>0.55</td>
<td>0.17</td>
<td>0.57</td>
</tr>
<tr>
<td>Chymotrypsinogen A</td>
<td>25,700</td>
<td>1.9</td>
<td>0.57</td>
<td>1.5</td>
</tr>
<tr>
<td>Apoferritin</td>
<td>16,500</td>
<td>1.2</td>
<td>0.35</td>
<td>0.65</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>17,200</td>
<td>1.4</td>
<td>0.43</td>
<td>0.74</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>14,300</td>
<td>1.1</td>
<td>0.32</td>
<td>0.46</td>
</tr>
<tr>
<td>RNase A</td>
<td>13,700</td>
<td>2.2</td>
<td>0.67</td>
<td>0.91</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>12,200</td>
<td>1.4</td>
<td>0.42</td>
<td>0.51</td>
</tr>
<tr>
<td>Trypsin inhibitor, lima bean</td>
<td>9,400</td>
<td>2.4</td>
<td>0.72</td>
<td>0.61</td>
</tr>
<tr>
<td>Insulin</td>
<td>5,700</td>
<td>2.8</td>
<td>0.83</td>
<td>0.24</td>
</tr>
</tbody>
</table>

* Molecular weights are from Dunker and Rueckert (4).

† The specific activities are expressed as millions of 14C dpn per mg of protein. The values were calculated assuming complete recovery of the protein from the Sephadex column.

‡ Acetylation densities are expressed as acetyl residues per 10^4 daltons of protein.

§ Values are calculated by multiplying the molecular weight of the protein (Column 2) by the corresponding acetylation density (Column 4).
tions. The densities of acetylation predicted by Equation 11 were similarly successful (Table I, compare Columns 6 and 7).

Because of variations in the composition and conformation of different proteins, their rates of reaction with acetic anhydride are, a priori, unpredictable. However, examination of Table I reveals that the acetylation densities of most proteins labeled under comparable conditions are similar to that of bovine serum albumin, within a factor of 2. The low acetylation densities of phosphorylase a, catalase, and carboxypeptidase A are probably due to the fact that they were acetylated as insoluble protein suspensions. This remarkable similarity implies that most proteins have similar densities of freely reactive groups and that the acetylation rate constants of most proteins are, in fact, rather similar. Thus Equations 10 and 11 should be applicable to a variety of other proteins, provided they are acetylated in 0.3 M sodium phosphate at pH 7.2. Alteration of these conditions requires experimental re-evaluation of the velocity constant ratio, $K/K_s$, characteristic of that buffer. Once this ratio is known for one protein, such as bovine serum albumin, the approximate acetylation efficiency (Equation 7) and acetylation density (Equation 8) can be computed for any other protein under the same conditions.

Suitability of Radioacetylated Proteins as Molecular Weight Calibration Standards in Sodium Dodecyl Sulfate-containing Polyacrylamide Gels and in Gel Filtration in 6 M Gdn-HCl—Since the initial impetus for developing an in vitro radiolabeling procedure was to produce radioactive calibration standards for molecular weight determinations by gel electrophoresis or gel filtration in 6 M Gdn-HCl, the mobility of the radioacetylated proteins was closely monitored in these systems. When the homogeneity of each radioacetylated protein was checked by electrophoresis in sodium dodecyl sulfate-containing gels, only the expected protein band(s) were detectable by staining or by analysis for radioactivity. Once the homogeneity of the radioacetylated proteins was established, the mobility of the radioactive proteins and of the corresponding unlabeled protein were compared using the split gel technique (4) which can detect subtle differences in protein mobilities. As shown in Fig. 4, no differences could be detected in the mobility of several of the $^14$C-acetylated proteins described in Table I and the corresponding unlabeled protein. Thus at the degree of acetylation employed, the mobilities of the radioacetylated proteins on sodium dodecyl sulfate-containing polyacrylamide gels was indistinguishable from those of the corresponding unlabeled protein.

Typical profiles obtained using $^14$C-acetylated markers in sodium dodecyl sulfate-gels and Gdn-HCl columns are presented in Fig. 5, Panels A and B, respectively. The standard curves (Panels C and D) calculated in both cases fall well within the expected accuracy for these techniques, and apparent molecular weights calculated using radioacetylated proteins as calibration standards have been in good agreement with other methods. The identity of the radioactivity found eluting with the DNP-alanine marker proteins on hydroxylapatite columns, and following the kinetics and products of protein cleavage by cyanogen bromide in 70% formic acid.

Acetylation of Avian Myeloblastosis Virus—In the course of studies on the structure and composition of avian oncornaviruses, we applied the acetylation procedure to intact and sodium dodecyl sulfate-disrupted AMV. Intact AMV was labeled with $[^3]$H]acetic anhydride, disrupted with sodium dodecyl sulfate and subjected to electrophoresis on sodium dodecyl sulfate-containing polyacrylamide gels to determine which viral proteins were labeled (see Fig. 6A). It has been established by others (22, 23) that the virus is constructed of a complex ribonucleoprotein core encased within a lipid envelope. The lipid envelope is known to include two externally located virus-specific glycoproteins, gp86 and gp35, as well as a number of more poorly defined proteins which migrate on gels between gp86 and gp35. The latter are thought to be derived from the host cell. Polypeptide chains p27, p19, p12, p15, and p10 are thought to be components of the internally located RNP core (22, 24). Identification of the peaks shown in Fig. 6, Panel A, was confirmed by coelectrophoresis with purified $^{14}$C-amino-acid-labeled Rous sarcoma virus in the same gel (not shown). It is known that the electrophoretic mobilities of the homologous polypeptides of these two viruses are very similar (23). It also was established in these comparative experiments that the dominant fast peak PL comigrates with the phospholipid peak of Rous sarcoma virus labeled with $[^3]$P]orthophosphate (24). Hence the AMV peak PL probably contains acetylated derivatives of phosphatidylethanolamine and phosphotidylserine which bear free amino groups reactive with acetic anhydride (25, 26).
FIG. 5. Mobility of $^{14}$C-acetylated calibration proteins during electrophoresis in sodium dodecyl sulfate-containing polyacrylamide gels and during gel filtration on 6% agarose beads in 6 M guanidine hydrochloride. A, profile of $^{14}$C-acetylated proteins subjected to electrophoresis on sodium dodecyl sulfate-containing polyacrylamide gels. About 7000 dpm each of the $^{14}$C-acetylated marker proteins phosphorylase a (1), bovine serum albumin (2), ovalbumin (3), chymotrypsinogen A (4), and myoglobin (5) were prepared and subjected to electrophoresis on a 10-cm gel of 10% acrylamide as described under "Materials and Methods." Electrophoresis was for 7 hours at 8 ma per gel. The gel then was sliced into 1-mm fractions using a Gilson gel fractionater and the radioactivity of each fraction was assayed in tT21. Migration of proteins is from left to right. B, gel filtration of $^{14}$C-acetylated marker proteins on agarose beads in 6 M Gdn-HCl. $^{14}$C-Acetylated bovine serum albumin (1), chymotrypsinogen A (2), lysozyme (3), and insulin (4) marker proteins were carboxymethylated and chromatographed as described under "Materials and Methods." Electrophoresis was for 7 hours at 8 ma per gel. The gel then was sliced into 1-mm fractions using a Gilson gel fractionater and the

It is known that RNA can be acetylated; however, high concentrations (0.3 to 1 M) of acetic anhydride are usually employed (27). To examine the reactivity of RNA under our reaction conditions, purified yeast ribosomal RNA was treated with acetic anhydride as described in Table III. Two proteins were radioacetylated under similar conditions in the same experiment for comparison. The results show that RNA was less than 1% as reactive as protein. In this regard, Rice and Means (13) have found that tobacco mosaic virus RNA is similarly unreactive with formaldehyde and sodium borohydride.

DISCUSSION

An advantage of labeling proteins by the acetylation procedure described here is that the reaction may be carried out at neutral pH and does not require the use of denaturing agents, immiscible organic solvents, or oxidizing or reducing agents. Thus, undesirable solvent-induced alterations to the protein are minimized. With limited acetylation, for example, 1 to 2 acetyl residues per $10^6$ daltons of protein, one would predict little alteration in the physiochemical or enzymatic properties of most proteins. Lysozyme, trypsin, and $\alpha$-chymotrypsin retain full enzymatic activity after extensive acetylation (19). Miller and Great (20) have confirmed this result and have shown further that RNase A and concanavalin A remain fully active after acet-
It is worth noting here that component p10 of AMV was radioacetylated and that viruses.

preparing high specific activity virus, labeled in both protein and cleaning resolved from the other core proteins (Fig. 6). This fact has not previously been recognized because AMV p10 stains poorly with Coomassie blue (21) which is usually used to detect proteins and lipid amino groups of the erythrocyte membrane in both whole red blood cells and intact ghosts. With mild reaction conditions and limited substitution, one would expect little alteration in the integrity and activity of radioacetylated structures. Studies on the infectivity of viruses labeled with acetic anhydride are now in progress.

The ability of acetic anhydride to penetrate freely throughout the membrane and ribonucleoprotein core of a complex virus suggests that the acetylation procedure should be applicable to labeling a variety of other biological structures. For example Carraway et al. (26) have used radioactive acetic anhydride to label the proteins and lipid amino groups of the erythrocyte membrane in both whole red blood cells and intact ghosts. With mild reaction conditions and limited substitution, one would expect little alteration in the integrity and activity of radioacetylated structures. Studies on the infectivity of viruses labeled with acetic anhydride are now in progress.

As the use of radioimmune assays has become more widespread, there has been an increase in the reports of problems associated with the radioiodination techniques commonly employed in these procedures (7). It would be surprising if limited acetylation under the reaction conditions described here appreciably alters the antigenicity of proteins. Radioactive acetic anhydride with specific activities 50-fold greater than that employed here are available commercially. Hence it seems likely that radioacetylation will provide a useful alternative technique for preparing labeled antigens and antibodies, especially in cases where a long half-life is required.

Acknowledgments—We thank the Cancer Virus Program of the National Cancer Institute, and Joseph W. Beard, for providing us with viremic plasma from leukemic chicks, and Dr. M. Nomura for the purified ribosomal RNA from yeast. The excellent technical assistance of Jane Hubert is also gratefully acknowledged.

REFERENCES

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Table III

Relative reactivities of protein and RNA with [3H]acetic anhydride

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Millions of [3H] dpm per mg&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Acetyl residues per 10&lt;sup&gt;4&lt;/sup&gt; daltons&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast ribosomal RNA</td>
<td>0.07</td>
<td>0.001</td>
</tr>
<tr>
<td>Carboxypeptidase A</td>
<td>7.3</td>
<td>0.13</td>
</tr>
<tr>
<td>Rovine serum albumin</td>
<td>14.0</td>
<td>0.26</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are calculated assuming complete recovery of the RNA or protein from the Sephadex column.

The purified yeast ribosomal RNA was ethanol-precipitated and dissolved to a final concentration of about 1% in 0.3 m sodium phosphate containing 1% sodium dodecyl sulfate by heating 2 min at 100°. A 0.5-ml sample of the 1% RNA then was treated with 5 pmol of [3H]acetic anhydride in 10 μl of dioxane (“Materials and Methods,” Stock C). After 30 min at room temperature, the RNA was desalted through a Sephadex column (1.4 × 25 cm) eluted with 0.01 m sodium phosphate, pH 7.2, containing 0.1% sodium dodecyl sulfate.

A 0.5-ml sample of 1% protein in 0.3 m sodium phosphate, pH 7.2, containing 1% sodium dodecyl sulfate was radioacetylated and purified as described above.

The acetylation procedure is a simple and inexpensive means of preparing high specific activity virus, labeled in both protein and lipid, for use in various structural studies. It is worth noting here that component p10 of AMV was radioacetylated and that it, like the homologous protein of Rous sarcoma virus (24), was cleanly resolved from the other core proteins (Fig. 6). This fact has not previously been recognized because AMV p10 stains poorly with Coomassie blue (21) which is usually used to detect AMV proteins on polyacrylamide gels.

A. G. Mosser, personal communication.

![Radioacetylation of intact (Panel A) and sodium dodecyl sulfate-disrupted (Panel B) avian myeloblastosis virus. Intact and sodium dodecyl sulfate-disrupted AMV preparations were radioacetylated under otherwise identical conditions as described under "Materials and Methods." Electrophoresis was performed on sodium dodecyl sulfate-containing polyacrylamide gels for 19 hours at 8 ma per gel. The protein nomenclature is based on the recommendations of the Oncornavirus Discussion Group held in New York City in June, 1973. The radioacetylated phospholipids (PL) accounted for about 60% of the counts applied to the gel; the protein accounted for the remaining 40%. The specific activities of the radioacetylated intact and sodium dodecyl sulfate-AMV preparations were about 1.0 × 10<sup>9</sup> dpm per mg of virus and 2.0 × 10<sup>9</sup> dpm per mg of virus, respectively. Bromophenol blue (BPR) was used as a visual tracking dye during electrophoresis. Protein migration is from left to right.](image-url)
Radiolabeling of proteins and viruses in vitro by acetylation with radioactive acetic anhydride.

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