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ON THE LABELLING OF OXIDIZED CELL SURFACE MEMBRANES BY ACYL HYDRAZIDES

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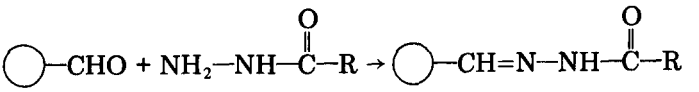
Summary

The covalent modification of bovine erythrocyte membranes by first oxidizing cell surface sugars followed by the covalent coupling of the oxidized components with hydrazides was investigated. The aldehydic groups were introduced either chemically, by mild periodate oxidation, or enzymically with galactose oxidase. The periodate (0.1 mM) and galactose oxidase (50 units) oxidations were complete at 5 and 240 min, respectively, in phosphate-buffered saline (pH 7.4) at 30°C using $2 \cdot 10^9$ cells $\cdot$ ml^{-1}. The rates of hydrazone formation between the oxidatively generated aldehyde groups and α-[^{3}H]acethydrazide were also measured (pH 7.4) at 30°C. The reactions with the periodate and galactosde oxidase-treated cells went to completion in 1.5 and 6 h, respectively. Aniline had a marked catalytic effect on these rates. With 5 mM aniline under the same conditions, the periodate-treated cells reacted to completion with acethydrazide in 30 min and the galactose oxidase-treated cells in 40 min. As expected, although the rates of α-[^{3}H]acethydrazide incorporation were increased with aniline, the extents of the reaction were not. Finally, the stability of the hydrazone linkages were measured. The hydrazone bonds formed when the galactose oxidase-treated cells were reacted with acethydrazide were completely stable at 30°C for 24 h whereas those formed from the periodate-treated cells were not. In the latter case roughly 50% of the membrane-bound hydrazone linkages were lost after 6 h at 30°C. The remaining 50%, however, were stable to cleavage. Thus, these sites are clearly heterogeneous in nature.

Abbreviation: *t*-Boc, *t*-butoxycarbonyl.

Introduction

The method of introduction of new haptens onto a cell surface by coupling a hapten containing acyl hydrazide with cell surface aldehyde groups is a generally used one. For example, biotin hydrazide [2],



sugar-containing hydrazides [3], 2,4-dinitrophenyl hydrazide [4], and methionine sulfone hydrazide [5], have all been covalently attached to the cell membrane by this technique. The cell surface aldehydic groups were generated by either chemically oxidizing surface sialic acid with sodium periodate or by enzymically oxidizing cell surface galactose moieties with galactose oxidase [6]. As this technique of hydrazide coupling is an important one and as very little is known about the chemistry of this process as applied to biological systems we sought to study the reactions involved and the chemical stability of the generated hydrazones. In this communication, the rates of reactions of tritiated acethydrazide (C^3H_3-CO-NH-NH_2) as the titrant with aldehydic groups generated at the surface of bovine erythrocytes by both periodate and galactose oxidase are reported. Aniline strongly accelerates the rate of hydrazide formation from cell surface aldehyde groups produced by both methods. Furthermore, the stability of the cell surface hydrazone moieties were measured and found to strongly depend on the method of initial oxidation.

Materials and Methods

Sodium metaperiodate was a product of Mallinckdrodt Inc (lot WYCD). The salt was dried in vacuo at 65°C for 8 h before weighing. *t*-Butyl carbazate and galactose oxidase from *Dactylium dendroides* (spec. act. = 180 units · mg^{-1}) were purchased from the Sigma Chemical Co. Sodium[^{3}H]acetate was product of New England Nuclear. Dicyclohexylcarbodiimide and aniline were products of the Aldrich Chemical Co. Bovine erythrocytes were collected in heparin phosphate-buffered saline (pH 7.4) from a local slaughterhouse on the day that they were used. Egg phosphatidylcholine-derived small unilamellar liposomes containing the synthetic galactosyl-containing glycolipid were prepared by the method of Barenholz et al. [1].

Syntheses

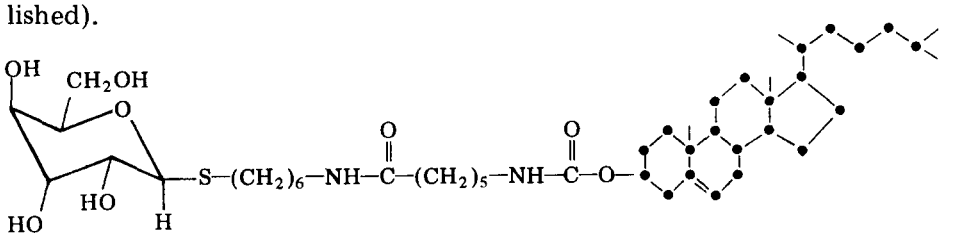
α-[^{3}H]Acethydrazide trifluoracetate salt. 25 mCi of sodium[^{3}H]acetate (specific activity = 9.4 Ci · $mmol^{-1}$; 0.22 mg in ethanol) was evaporated to dryness in a 20 ml flask. 300 mg acetic acid (5 mmol) was added in 10 ml dry methylene chloride along with 660 mg (5 mmol) *t*-butyl carbazate and 1 g (5 mmol) dicyclohexylcarbodiimide. The solution was stirred overnight at room temperature and the white precipitate of dicyclohexylurea was filtered off. The methylene chloride was evaporated off to afford the *t*-Boc-protected acethydrazide. The white solid was recrystallized from ether/hexane (m.p. 111—112°C) NMR (*S* at 1.5 ppm (nine protons), *S* at 2.05 ppm (three), and *M*

at 7.2 ppm (two) C^2HCl_3 solvent. This compound was cooled down in a 10-ml flask (ice/water) and 2.5 ml trifluoroacetic acid was added. Immediate bubbling was observed. After 0.5 h at 5° C and 2 h at room temperature the trifluoroacetic acid was removed and the resulting oil was tritiated with dry ether to yield the solid white, non-hygroscopic product. After drying, 532 mg of pure trifluoroacetic salt of α-[^{3}H]acethydrazide was obtained (m.p. 96—97° C), yield 57% based on acetic acid. Specific activity = 2.65 Ci · mmol^{-1}.

Anal. Calc. C: 25.53%; H: 3.72%; N: 14.89%
Anal. Found. C: 26.27%; H: 3.22%; N: 14.65%.

TLC analysis of the α-[^{3}H]acethydrazide shows a single spot at $R_F = 0.22$ (chloroform/methanol; 9 : 1) on EM silica plates (aluminum back) 95% of the radioactivity runs with this spot. When treated with benzaldehyde, a new spot with $R_F = 0.73$ is obtained (hydrazone). Again 95% of the radioactivity runs with this new spot.

^{35}*S-labelled 6-(6-aminohexanamido)hexyl-1-thio-β-D-galactopyranoside cholesterol.* This galactosyl-cintaining cholesterol analog was prepared by a method to be published (Rando, R.R., Orr, G.A. and Bangerter, F.W., unpublished).



Erythrocyte preparation. The erythrocytes were repeatedly washed with ice-cold phosphate-buffered saline (pH 7.4) and spun down (2000 × *g*) three times or more. The buffy coat was removed by aspiration. The cells were continuously washed until no more white layer appeared. At this point the cells were ready for modification.

Results and Discussion

Freshly prepared bovine erythrocytes in phosphate-buffered saline (pH 7.4) were treated with either 0.1 mM periodate for 5 min at 30° C or with 50 units of galactose for 2 h. The cells were then spun down and washed three times with phosphate-buffered saline. Erythrocytes oxidized by these techniques were treated with 5 mM of α-[^{3}H]acethydrazide (C^3H_3-CO-NH-NH_2) as its trifluoroacetate salt in phosphate-buffered saline. This reagent quantitatively measures the amount of liberated aldehyde groups. Quantitative studies cannot be done with sodium borotritide because the isotope effects on the reduction step are unknown. There is, of course, no isotope effect on the reaction rates when using the labelled acethydrazide. At various times cells were removed and washed three times with phosphate-buffered saline. Ghosts were prepared from these cells and were then counted. In Fig. 1A and B the rates of hydrazone formation are shown with periodate and galactose oxidase-treated cells, respectively. As indicated in this Fig. 1, the rates of the acethydrazide reactions are

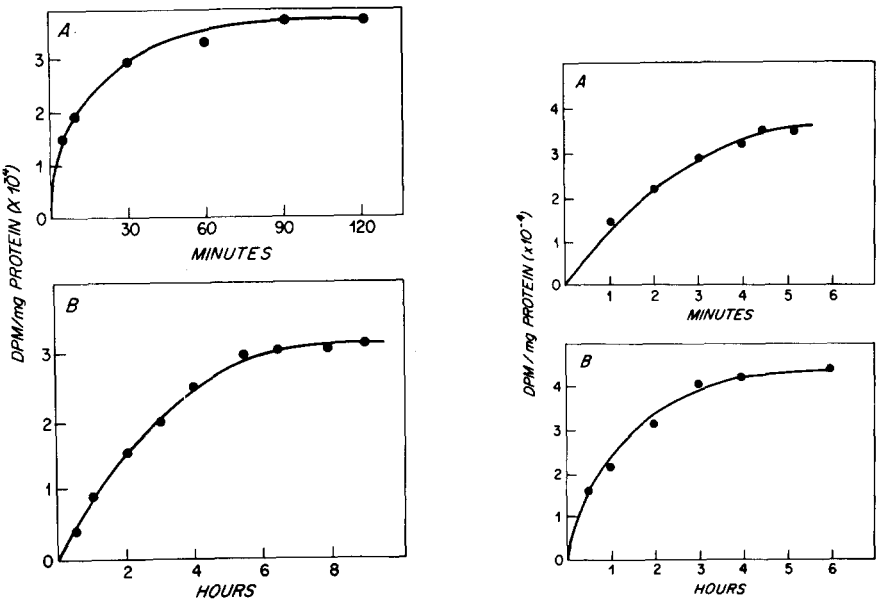


Fig. 1. (A and B) Titration of cell surface aldehyde group generated by periodate and galactose oxidase. (A) periodate-treated cells. (B) galactose oxidase-treated cells. $4 \cdot 10^9$ erythrocytes $\cdot$ ml^{-1} (Coulter counter determination) in phosphate-buffered saline were prepared as indicated in Materials and Methods. These erythrocytes were treated either with sodium periodate at 0.1 mM for 5 min at 30°C or 2 h with 50 units $\cdot$ ml^{-1} galactose oxidase and 10 units catalase. After treatment, the cells were washed three times with 10 ml phosphate-buffered saline and spun down. The cells were then treated with 5 mM [^{3}H]-acethydrazide trifluoroacetate salt (specific activity = 2.65 Ci $\cdot$ M^{-1}) with stirring at 30°C. At the indicated times 1 ml of the mixture was removed and diluted with 10 ml phosphate-buffered saline. The cells were spun down and washed three times with the phosphate-buffered saline. The cells were then lysed in 5 mM of sodium/potassium phosphate buffer (pH 7.4). The ghosts were then isolated by the method of Dodge et al. [10], and aliquots were counted in Aquasol. The amount of protein in the ghosts was determined by the Lowry method [11]. The samples were done in triplicate and averaged. In the above graphs, the amount of radioactivity incorporation is expressed as dpm $\cdot$ mg^{-1} protein. In the absence of oxidant no radioactivity (less than 100 dpm $\cdot$ mg^{-1} protein) was incorporated with the ghosts after [^{3}H]acethydrazide incubation.

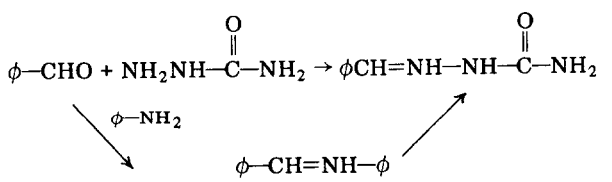
Fig. 2. (A and B) Time course for the periodate and galactose oxidase reactions. (A) Periodate-treated cells. (B) Galactose oxidase-treated cells. As in Fig. 1, bovine erythrocytes were incubated with 0.1 mM periodate or 50 units galactose oxidase (+ catalase) at 30°C. Catalase is added because the galactose oxidase is inactivated by the peroxide it liberates. At the indicated times 1 ml samples were removed and washed several times. These cells were then treated with 5 mM α-[^{3}H]acethydrazide for 12 h and thoroughly washed. Ghosts were prepared and counted in Aquasol. All results are the average of triplicate assays.

relatively slow at pH 7.4. Insofar as the pH can be varied with cells (pH 6.5–8), no significant increase in the rate of hydrazone formation was observed. The experiments shown in Fig. 1 determined the amount of time required for the acethydrazide reaction to go to completion. This information could then be used to measure the rates of the oxidation reactions by α-[^{3}H]acethydrazide titrations of the aldehyde groups. In Fig. 2, the time courses for the periodate and galactose oxidase reactions are shown. In Fig. 2A and B the rate of the periodate and galactose oxidase reactions are shown. The periodate reaction is clearly the much more rapid of the two. From the amount of ^{3}H incorporation in the periodate-treated cells, it can be calculated that a minimum of 20%

of the added periodate is utilized to generate titratable aldehyde groups. It is possible that this low yield is observed because some of the periodate enters the cell and oxidizes cytoplasmic elements that would not be counted by our technique. However, it has been reported that periodate only penetrates erythrocytes very poorly at low temperatures [7]. It should be noted that the overall yield of approximately 20% would be obtained if each of the individual yields (periodate oxidation, hydrazide formation, and ghost preparation) were 60%. Under the conditions of these experiments, the periodate preferentially cleaves the glycol side chain of sialic acid [7]. The oxidation of this grouping is linear with the amount of periodate added up to at least 1 mM so that substrate (cell surface sialic acid) is not limiting here. Furthermore, the fact that linearity is found insures that periodate is not being preferentially wasted at lower concentrations.

In the galactose oxidase experiments reported in Fig. 2B, it is clear that the amount of titratable aldehyde groups levels off after approximately 4 h. Neither higher concentrations of galactose oxidase nor longer incubation times increased the yield of tritium incorporation after labelling with α-[^{3}H]acethydrazide. Not all of the cell surface galactose moieties have been oxidized by this technique since untreated erythrocytes and those treated with galactose oxidase (to completion) followed by reaction with acethydrazide are both equally susceptible to agglutination by the castor bean agglutinin at 32 $\mu g \cdot ml^{-1}$. This lectin is specific for β-galactoside moieties [8]. Thus, galactose oxidase does not appreciably oxidize those galactose residues recognized by the castor bean agglutinin.

Since the rates of hydrazone formation are slow, ways to increase the velocity of this reaction were sought. As previously mentioned, a full pH rate profile for this reaction is not possible with cells due to their limited stability at pH extremes. Thus, a catalytic agent other than acid or base was sought. From model studies, it had been determined that aniline catalyzed the rate of hydrazone formation between aldehydes and semicarbazide [9]. The presumed mechanism here involves the rapid formation of a reactive Schiff base between the aldehyde and aniline followed by the facile displacement of the aniline moiety by the semicarbarzide. In this instance, the aniline can be considered to be an agent of covalent catalysis.



For these reasons, aniline was tested as a catalyst in the formation of the hydrazone from [^{3}H]acethydrazide and cell surface aldehyde groups generated by either galactose oxidase or by periodate. As can be seen in Fig. 3a and b aniline has a marked accelerative effect on the rates of hydrazone formation in both instances. The rates increase with increasing aniline concentration up to the highest concentration (5 mM) tested. Generally, the rates of hydrazone formation with the 6-dehydrogalactose (galactose oxidase product) are slower

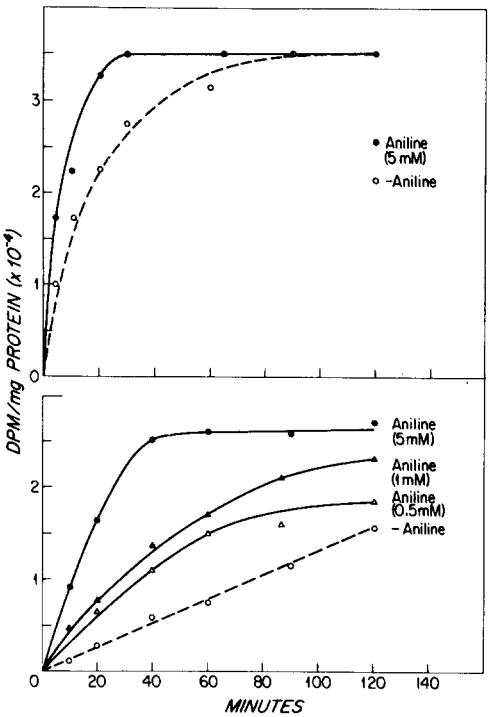


Fig. 3. Catalysis of hydrazone formation with aniline. (A) Periodate-treated cells. Washed and prepared erythrocytes ($4 \cdot 10^9$ cells $\cdot$ ml^{-1}) in phosphate-buffered saline were treated with 0.1 mM sodium periodate for 15 s at 30°C. The cells were then washed three times with phosphate-buffered saline and treated with 5 mM α-[^{3}H]acethydrazide in the presence and absence of 5 mM aniline. At various times 1 ml of cells were removed, washed three times with phosphate-buffered saline, lysed and the amount of radioactivity present in the ghosts was determined. Each point in the above curve is the average of three experiments. (B) Galactose oxidase-treated cells. Washed and prepared erythrocytes ($4 \cdot 10^9$ cells $\cdot$ ml^{-1}) in phosphate-buffered saline were treated with 50 units $\cdot$ ml^{-1} galactose oxidase and the 10 units $\cdot$ ml^{-1} catalase in phosphate-buffered saline for 4 h. The cells were washed three times in phosphate-buffered saline and incubated with 5 mM α-[^{3}H]acethydrazide in the presence of 5 mM aniline and in its absence. At various times 1 ml of cells were removed, washed three times with phosphate-buffered saline, lysed and the amount of radioactivity present in the ghosts was determined. Each point in the above curve is the average of these experiments.

than with the periodate-liberated aldehyde groups and hence, the rate acceleration achieved with aniline are found to be more important here. No significant difference in the actual amount of aldehydes titrated are observed in the uncatalyzed versus the catalyzed cases. After 12 h in the galactose oxidase case, as many counts were incorporated in the uncatalyzed control as in the catalyzed example. We have further checked the galactose oxidase results in model systems. Small unilamellar liposomes were prepared from pure egg phosphatidylcholine and a β-D-galactose-containing cholesterol analog. The cholesterol analog is expected to distribute randomly in the bilayer like cholesterol itself. It could be shown indeed that galactose oxidase could oxidize to completion in 3 h approximately 60% of the added galactose (Rando, R.R., Orr, G.A. and Bangerter, F.W., unpublished results). The remaining galactose was presumably on the inside of the bilayer inaccessible to the enzyme. The rates of hydrazone formation between galactose oxidase-treated (3 h) liposomes with

α-[^{3}H]acethydrazide are shown in Fig. 4. Again, a strong accelerative effect of aniline is noted and again the effect is only on the rate of hydrazone formation and not the extent. It is of interest to note that it has been reported that manganese chloride will increase the extent of the reaction between methionine sulfone hydrazide and aldehydes in phosphate-buffered saline [5]. In our hands, manganese phosphate precipitated out at pH values of 6—8 so that we were not able to repeat these studies.

A further point to investigate here is the stability of the hydrazone bonds once formed. Clearly, it would be highly advantageous if these bonds were stable to hydrolysis in membrane modification studies. To these ends, erythrocytes were oxidized with periodate or galactose oxidase and then reacted with α-[^{3}H]acethydrazide. The cells were thoroughly washed with phosphate-buffered saline and then allowed to remain at 30°C in phosphate-buffered saline (pH 7.4). At various times cells were removed, washed several times, and ghosts were prepared and counted. The results of this study are shown in Fig. 5. It is clear that the hydrazones formed using the two oxidation techniques differ markedly in stability. In the galactose oxidase case the hydrazone bonds were quite stable to hydrolysis. Even after 24 h, no cleavage was

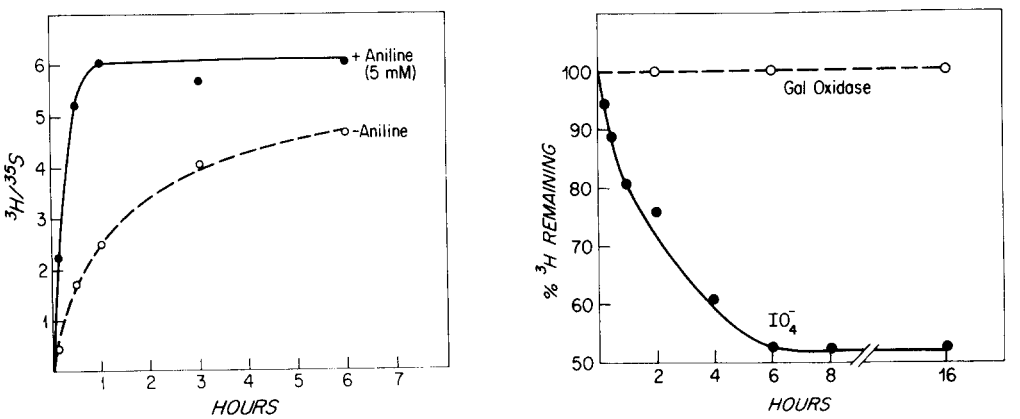


Fig. 4. α-[^{3}H]Acethydrazide titration of galactose oxidase-treated galactosyl glycolipid-containing liposomes. Small unilamellar liposomes (average diameter = 400 Å) containing 10% ^{35}S-labelled 6-(6-aminohexanamido)hexyl-1-thio-β-D-galactopyranoside cholesterol (specific activity = 0.23 Ci · M^{-1}) containing liposomes were oxidized with 450 units of galactose oxidase at 22°C for 20 h. The control consisted of untreated liposomes. The oxidized liposomes were then treated with 5 mM α-[^{3}H]acethydrazide (specific activity = 2.65 Ci · M^{-1}) in the presence of 5 mM aniline and in its absence. At the indicated times duplicate 0.25 ml samples were removed from each of the tubes and immediately dialyzed against 10^3 vols. phosphate-buffered saline in the cold. After three buffer changes, the liposomes were removed and the ^{3}H/^{35}S ratios were determined. The limiting ratio of approximately 6 occurs because all of the available galactose in the external face of the small liposomes has been oxidized. After 12 h the untreated (aniline) sample attains the same ratio. Samples untreated with galactose oxidase do not incorporate ^{3}H in the presence or absence of aniline.

Fig. 5. Stability of hydrazone bonds. Bovine erythrocytes were oxidized with 0.1 mM periodate or 50 units · ml^{-1} of galactose oxidase as in Fig. 1. The cells were then labelled with 5 mM [^{3}H]acethydrazide as before. After thorough washing the cells were then agitated in phosphate-buffered saline in 30°C. At varying times samples were removed, thoroughly washed, and ghosts were prepared. The remaining ^{3}H in the ghosts, corrected for protein concentration, was then determined. The above plot shows the percentage of radioactivity remaining after incubation. •, periodate-treated cells, and ○, galactose oxidase-treated cells.

detected. On the other hand, the periodate method yielded hydrazone bonds of more limited stability. After 6 h at 30°C, 40—50% of the radioactivity was lost. However, the remaining 50% of the hydrazide moieties were bound in a stable fashion. Thus, at least two classes of hydrazone bonds exist here, the second of which is stable to cleavage. Whether the instability results from the increased hydrolysis of the hydrazone or cleavage of the derivatized sialic acid is not known at the present time. However, it is certain that when using the periodate modification technique followed by treatment with a hydrazide, the modified cells should be immediately used to obtain reproducible results.

The introduction of new chemical groups on membranes by oxidation followed by hydrazone formation is a useful one for several reasons. Primarily, the techniques, whether they may be chemical or enzymic, are mild and relatively specific for sialic acid and the galactose, respectively. It should be pointed out that the specificity of the periodate reaction is, of course, more suspect, but under the conditions of the reactions used it is reported to only oxidize sialic acid [7]. In addition to the favorable oxidative techniques, hydrazone formation is also a positive aspect of this technique since the pK_a values of the imine nitrogen of these compounds is in the 3—4 range. Therefore, the membrane charge is not altered. This is not the case with many modifying agents. For example, acylating agents increase the net negative charge of the membrane when they react with the surface amines. In this article we have shown that the aldehyde groups generated by the two oxidative techniques differ both in reactivity towards the acylhydrazide and in the stability of the formed hydrazone. It is clear that the rate accelerations observed in the presence of aniline are most important when used in conjunction with the galactose oxidase technique where subsequent hydrazone formation is exceedingly slow. Although we have no data to support this, aniline at high concentrations might be toxic in the cells since the material should easily penetrate cell membranes. If it is proved to be the case, this potential problem could be circumvented by using a negatively charged aniline derivative such as *p*-aminobenzenesulfonic acid or *p*-aminobenzenephosphorous acid. Neither of these reagents should cross the membrane. The relative lack of stability of the hydrazone formed when periodate is used as the oxidizing agent is problematic. Experiments done with this modification technique must be done rapidly. It should be mentioned here that the hydrazone is much more stable at ice/water temperature. At 10°C only approximately 15% of the radioactive acethydrazide was lost after 18 h. Thus, the periodate oxidation technique is still usable if attention is paid to the limited stability of the hydrazone bonds formed.

Acknowledgements

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