Changes in the Molecular Structure of Axonal and Red Blood Cell Membranes Following Treatment with Phospholipase A_2*

Henry Simpkins,† Sin Tay, and Elaine Panko

ABSTRACT: Phospholipase A_2 treatment of erythrocyte membranes resulted in no detectable release of protein and fatty acids, but between 5 and 10% of the lipid phosphorus was released from the membranes. The overall helical configurations of the membrane proteins as determined by circular dichroism measurements appeared unchanged. Investigation by the approach of spin labeling showed that the lipid regions of the membrane became more immobile and ordered, and the environments of certain membrane sulfhydryl groups appeared altered after phospholipase A_2 treatment. Similar molecular changes in the structure of the lipid regions were found in both red cell and the nonmyelinated nerve membranes. A possible explanation of the observed changes in the lipid regions is that, after enzymatic cleavage of the β-ester group, a condensation of the lipid fatty acid chains results in the formation of a more closely packed lipid array. Changes are also observed in the conformation of some of the membrane proteins as shown by changes in their *N*-ethylmaleimide spin-label spectrum. Treatment of the membrane with lysolecithin (10% of lipid weight) results in changes in the *N*-ethylmaleimide spin-label spectrum that is similar to those produced by the phospholipase A_2 and opposite to those produced by treatment of the membrane with 0.2% sodium dodecyl sulfate or 2% lysolecithin. Treatment by the latter produces an increase in the mobile component of the spin label at the expense of the immobilized component. Also phospholipase A_2 and lysolecithin treatments result in similar changes in the polyacrylamide gel patterns of the membrane proteins of the red blood cells. It therefore appears that enzyme treatment produces changes in the lipid conformation and specific changes in the protein conformation. The latter may solely be a result of lysophosphatidyl compounds binding to some membrane proteins, or binding *plus* changes produced by the conformational changes occurring in the lipid array.

The action of the enzyme phospholipase A_2 on phospholipids produces lysophosphatidyl compounds and fatty acids. It has been generally assumed that the lyso compounds so formed react with the membranes in much the same way as a detergent. It has been shown that endogenous lysolecithin will solubilize membrane protein (Blomfield *et al.*, 1966). Also phospholipase A_2 treatments, in the presence of added lecithin, produce rapid lysis of red cells, whereas the action of phospholipase A_2 by itself results in no lysis (W. B. Campbell, unpublished observations). These latter results however suggest that the detergent action of the enzyme requires exogenous phospholipid. It was found that enzyme treatment of the RBC[1] membrane does result in the release of some lysophosphatidyl compounds (5–10% of the total phospholipid) but not in the release of free fatty acids as the decrease in total lipid paralleled that of lipid phosphorus.

The action of phospholipase A_2 results in a greater decrease in the Ca^{2+}-activated and (Na + K)-transport ATPase activities than that caused by treatment with phospholipase C (Wallach, 1969) with Ehrlich ascites carcinoma plasma membrane preparations. A similar difference in enzyme activities is apparent with the (Na + K)-transport ATPase of the squid retina axonal membrane after treatment with phospholipases A_2 and C (Fischer *et al.*, 1970). Binding of glucagon to rat liver plasma membranes is reduced by 62% by phospholipase A_2 as compared to 33% with phospholipase C treatment (Tomasi *et al.*, 1970). A study as to the structural changes produced by phospholipase A_2 treatment of plasma membranes was therefore undertaken.

The technique of spin labeling (McConnell and McFarland, 1970; Griffith and Waggoner, 1969) was chosen to study conformational changes occurring in the phospholipid regions and changes in the environments of the protein sulfhydryl groups. Nonmyelinated nerve bundles were employed in addition to RBC membranes as the former possess membrane axes orientated in a known direction. The approach relies on the fact that a spin-labeled nerve results in two sets of resonance characteristics depending on whether its axis is parallel or perpendicular to the magnetic field (Hubbell and McConnell, 1969b). That differences exist between these two esr spectra shows that the axis of the spin label is held in some defined position relative to the axonal axis. It can also be inferred that some ordered phospholipid structure is probably present (possibly a bilayer). A more detailed knowledge as to the precise structural changes occurring in the lipid regions of the membrane after enzyme treatment can thus be obtained with an orientated nerve membrane rather than with randomly orientated RBC membrane preparations.

The overall helical conformation of the membrane proteins was also monitored by circular dichroism. In addition, changes in the molecular weight distribution pattern of the membrane proteins were investigated employing detergent polyacrylamide gel electrophoresis techniques.

Materials and Methods

RBC membranes were prepared from outdated human blood of varying blood groups by the method of Dodge *et al.* (1963).

Walking leg nerve bundles (3–4 cm long; 1–3 mm in diameter) from the lobster *Homarus americanus* were excised and separated in lobster Ringers solution (Mendelson, 1969) at 4°.

* From the Lady Davis Institute for Medical Research of the Jewish General Hospital, Montreal, Quebec. *Received May 4, 1971.* Supported by the Canadian and Quebec Medical Research Councils.

† To whom to address correspondence.

[1] Abbreviations used are: RBC, red blood cell; NS, nitroxide stearate; BSA, bovine serum albumin; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate.

TABLE I: Compositional Data of RBC Membrane Preparation before and after Phospholipase A_2 Treatment.[a]

	Total P (μg/mg of Protein)	Total Fat (mg)/mg of Membrane Protein	Total Fat (mg)/mg of Membrane Protein Present after Treatment with Albumin–Spin-Label Complex
Control RBC membranes	29 ± 2.0	0.50 ± 0.05	0.45 ± 0.05
Phospholipase A treated membranes	25 ± 2.0	0.45 ± 0.05	0.41 ± 0.05

[a] The absolute values presented here vary from preparation to preparation, but a decrease in total phosphorus and fat of 5–10% on enzyme treatment is typical. The membranes were dialyzed exhaustively against 10 mM Tris-HCl (pH 7.4) before the phosphorus determinations were performed.

Naja Naja venom (Sigma Chemical Co., St. Louis), a source of phospholipase A_2, was dissolved in 0.01 M citrate buffer (pH 5.5) at a concentration of 0.1 mg/ml (0.3–0.5 mg/ml for the nerve bundle experiments), heated to 100° for 8 min, and filtered through a sintered-glass filter followed by a Millipore filter (0.45 μ), and the pH was adjusted to 7.4 with 0.05 M disodium hydrogen phosphate. $CaCl_2$ (0.2 M) was added to make the final enzyme solution 2 mM in Ca^{2+} ion. This step was omitted with nerve preparations because of their high intracellular calcium ion concentration.

The final enzyme solution was found to contain less than 0.1% (w/w) total phosphorus and to possess no proteolytic activity as determined by the release of ninhydrin-positive material after treatment of the erythrocyte ghosts. Also, polyacrylamide gel electrophoresis patterns of the treated and untreated ghosts (performed according to Lenard, 1970) showed no changes which were obviously caused by proteolysis (see Discussion).

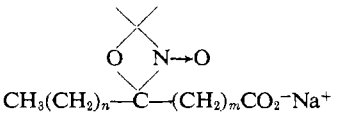
Erythrocyte ghosts (~3 mg of membrane protein/ml) were treated with enzyme solution in the ratio of 2:1 (v/v) for 30 min at 37°, and the reaction was stopped by centrifugation at 25,000*g*. Nerve bundles (containing ~2 mg of total protein) were treated with 4 ml of the enzyme solution (prepared as detailed above) for 1 hr at 37°. Control samples of ghosts or nerve bundles were treated in an identical fashion except for the omission of the enzyme.

Lysolecithin treatment was performed by incubation of lysolecithin (1.5 mg) (from egg lecithin, Sigma Chemical Co., St. Louis) in 0.2 ml of Ca^{2+}–citrate–phosphate buffer with 4 ml of the RBC membranes (4 mg of protein/ml) for 30 min at 37°. The lysolecithin was removed by washing in a similar manner to that employed with phospholipase A_2.

Phospholipids were extracted from the treated and control RBC membranes by the methods of Rose and Oklander (1965) or Folch *et al.* (1957). The nerve bundles were minced and homogenized in Ringers solution and the phospholipids extracted by the method of Folch *et al.* (1957). The phospholipid extracts were then evaporated to dryness and the number of intact ester linkages was determined by the method of Augustyn and Elliot (1969). Generally, 55–70% of the β-ester bonds in the erythrocyte ghosts and 40–50% of the corresponding bonds in the nerve membranes were cleaved by this enzyme treatment.

Protein and total phosphorus determinations were determined according to Lowry *et al.* (1951) and Bartlett (1959), respectively. Total lipid was determined by dichromate oxidation followed by titration of the excess dichromate by thiosulfate as described by Bloor (1928).

The *N*-ethylmaleimide and iodoacetamide spin labels were bought from Syva Associates (Palo Alto, Calif.). The fatty acid spin labels employed were the 12-NS (n = 5;



m = 10), 7-NS (n = 10; m = 5), and 5-NS (n = 12; m = 3). The 12-NS was synthesized according to Waggoner *et al.* (1969); the 5-NS, from the 5-ketostearic acid prepared according to Jones (1947); and the 7-NS, from the 7-ketostearic acid prepared according to Hunig and Eckardt (1962), as modified by O. H. Griffith (private communication, 1970).

The erythrocyte ghosts were spin labeled with the sulfhydryl specific reagents by stirring the membranes and the label in 20 mOsm phosphate buffer (pH 7.4) in the ratio of 4:1 w/w for 3 hr at 23°. Solutions of the fatty acid spin labels in ether were evaporated to dryness under a stream of nitrogen and then stirred for 12 hr with a 5% solution of BSA (fatty acid poor from Calbiochem) in 20 mOsm phosphate pH 7.4 buffer if RBC membranes were being labeled, or in lobster Ringers solution if the nerve bundles were being labeled. The resulting albumin–spin-label complex was filtered with a Millipore filter (0.45 μ) and exchanged with erythrocyte ghosts overnight at 4° or with nerve bundles for 1 hr at 4°. The ratio of albumin–spin label complex to RBC membranes was 1:7 (v/v), and with the nerve bundles normally 2–3 ml of spin label–albumin solution was used per nerve bundle. After treatment with protein or fatty acid spin labels, the erythrocyte ghosts were washed with 20 mOsm phosphate buffer pH 7.4 by repeated centrifugation at 25,000*g* for 15 min. The nerves were washed with the Ringer's solution.

Electron spin resonance spectra were obtained with a Jeolco MES-ME-1X spectrometer at 20–25°. A rectangular liquid cell was used for eryhrocyte ghost suspensions and a tissue cell for the nerve bundles.

Circular dichroism spectra were obtained with a Jasco-Durrum CD-5 with SS-20 modification spectropolarimeter in cells with 1-mm path length, at 20–25°. The spectra were placed on a molar absorptivity basis by using a mean residue molecular weight of 114 and a protein concentration determined according to Lowry *et al.* (1951).

Results

In Table I are presented data showing that the total amount of membrane phosphorus and total lipid fat decreases 5–10% on enzyme treatment. This means that most of the lysophosphatidyl compounds and probably all of the fatty acids remain associated with the membrane after enzyme treatment. A report by Fiehn and Hasselbach (1970) showed that washing of a phospholipase A_2 treated sarcoplasmic reticulum membrane with BSA resulted in the fatty acids, hitherto associated with the membrane, being absorbed by the albumin. In order

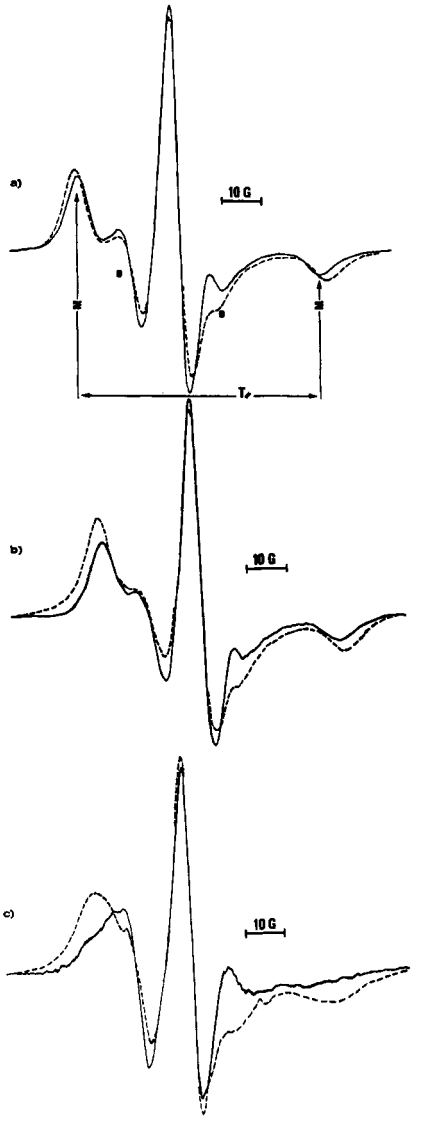


FIGURE 1: The RBC membranes labeled with the fatty acid spin labels: (a) the five nitroxide stearate labeled membranes before (———) and after (- - -) treatment with phospholipase A_2; (b) the seven nitroxide stearate labeled membranes before (———) and after (- - -) treatment with enzyme; (c) the twelve nitroxide stearate labeled membranes before (———) and after (- - -) enzyme treatment.

to determine whether the BSA–spin-label complex removed any fatty acid or lipid from the phospholipase A_2 treated membrane, total fat determinations were performed on the treated and normal membrane after the spin labels had been introduced, and as can be seen in Table I, little to no change was observed.

In Figure 1 are presented the fatty acid spin-label spectra before and after enzyme treatment of the RBC membranes. The 5-NS spectrum (where the nitroxide group is situated at about 5 Å from the charged carbonyl group) is presented in Figure 1a and is seen to possess an outer doublet (Σ) which arises because of the anisotropic motion of the nitroxide about the long axis of the fatty acid. It can be seen that, on enzyme treatment, the amplitude of these outer extrema (Σ) increases at the expense of the inner (θ). This means that the motion of the spin label has become even more immobile and anisotropic after enzyme treatment. A similar effect is found

TABLE II: The Mean Angular Deviation ($\cos^{-1}(\gamma^2)^{1/2}$) between the Spin Hamiltonian of Each Nitroxide u with the Long Axis v of Its Fatty Acid Chain.[a]

Membrane Prepn	Spin Label Employed	$T_{\|\|}$ (G)	Mean Angular Dev between v and u (deg)
RBC (control)	5-NS	29.3 ± 0.1	17.0 ± 0.5
RBC (treated)	5-NS	30.6 ± 0.1	11.0 ± 0.5
RBC (control)	7-NS	28.7 ± 0.1	21.0 + 0.5
RBC (treated)	7-NS	29.4 ± 0.1	18.5 ± 0.5
Axonal (control)	5-NS	28.0 ± 0.1	21.5 ± 0.5
Axonal (treated)	5-NS	29.5 ± 0.1	16.0 ± 0.5
Axonal (control)	7-NS	27.3 ± 0.1	25.0 ± 0.5
Axonal (treated)	7-NS	27.9 ± 0.1	23.5 ± 0.5

[a] The hyperfine splittings ($T_{||}$) between the outer doublets (Σ) are also presented.

with the 7-NS where the nitroxide group is situated further away from the charged carbonyl group (~7 Å) and is therefore more mobile and less anisotropic than the 5-NS. The 12-NS spectra in an untreated RBC membrane is presented in Figure 2c and exhibits no outer doublet as the nitroxide is now too far from its anchoring charged carbonyl group (14–15 Å) for its motion to be anisotropic. Enzyme treatment however produces enough immobilization of the nitroxide group for its motion to result in an esr spectrum possessing distinct extrema, *i.e.*, anisotropic motion.

Assuming that the motion about the long axis v of the fatty acid label is rapid and anisotropic, Hubbell and McConnell (1969a) showed that the mean deviation ($\cos^{-1}(\gamma^2)^{1/2}$ of the static spin Hamiltonian u with v can be determined and is a measure of the mobility of the nitroxide in a particular environment. If it is assumed that the static Hamiltonian has axial symmetry about its hyperfine axis, then the isotropic splitting constant, a_N, approximates to $^1/_3(T_x + T_y + T_z)$, where $T_x \simeq T_y \simeq T_\perp^m$ and $T_z \simeq T_{||}^l$, where $T_{||}^l$ is the hyperfine splitting constant of the nitroxide in a completely immobile environment (for example, in BSA or in a crystal). a_N was determined for each nitroxide from its spectrum in ethanol, and from this, $T_\perp^m$ was calculated. $T_{||}$ was then measured for a particular spectrum and γ^2 was calculated by the equation (Hubbell and McConnell, 1969a,b): $T_{||} = T_\perp^m(1 - \gamma^2) + \gamma^2 T_{||}^l$.

The mean angular deviation $\cos^{-1}(\gamma^2)^{1/2}$ gives an idea of the relative immobilization of the nitroxide group. It can be seen in Table II that after enzyme treatment this parameter is significantly decreased. It is also apparent that, as the nitroxide becomes closer to its anchoring charged carbonyl group, so the angular deviation also decreases (*cf.* 5-NS with 7-NS) showing that the nitroxides' motion becomes more anisotropic as it moves nearer the charged carbonyl group. Also it appears that the lipid regions of the axonal membrane are more mobile than those in an erythrocyte ghost as the angular deviation is greater for each spin label in a nerve membrane than that observed with the same label in an RBC membrane. Enzyme treatment of both plasma membranes results in a decreased mobility of the labels in the treated lipids which occurs all along the fatty acid chains of treated phospholipids

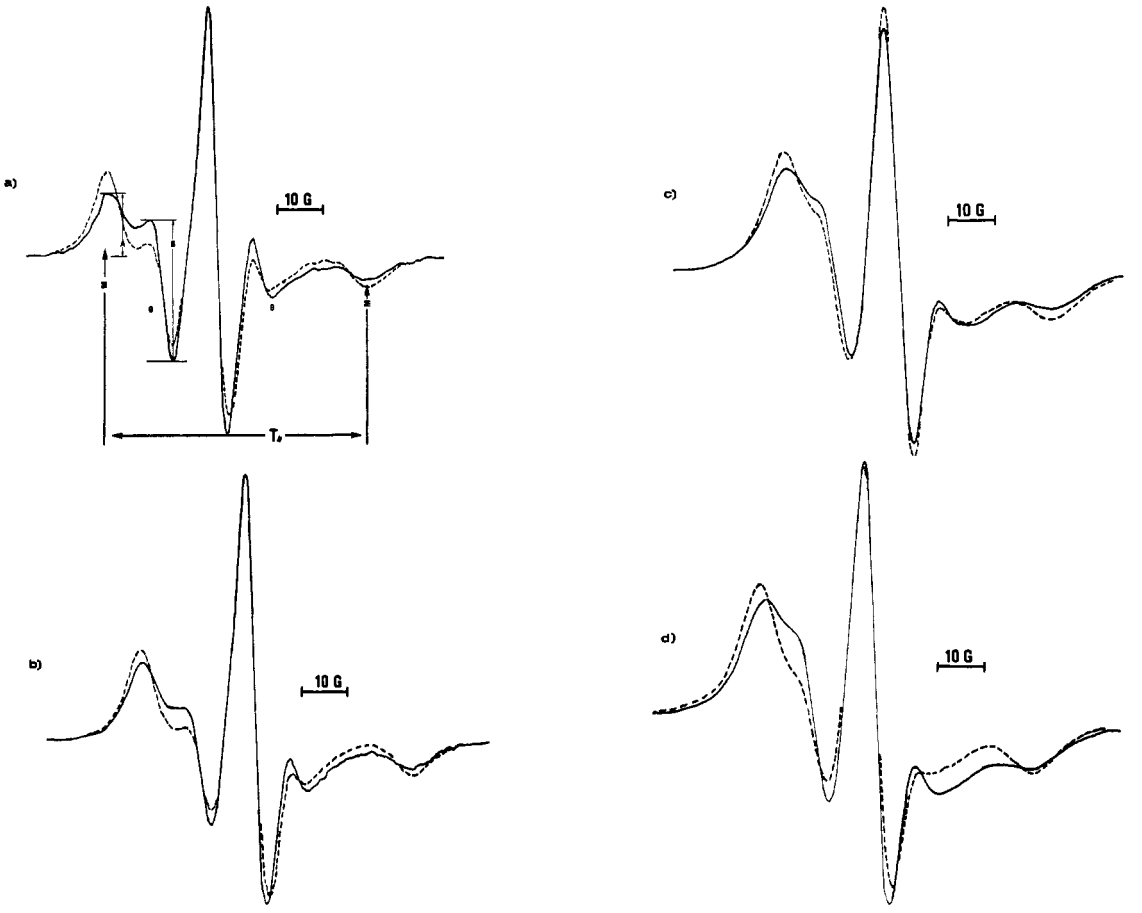


FIGURE 2: The nerve membranes labeled with the following fatty acid spin labels: (a) 5-nitroxide stearate with the axonal axis parallel (———) and perpendicular (– – –) to the applied magnetic field; (b) 5-nitroxide labeled membrane *after enzyme treatment* with its axonal axis parallel (———) and perpendicular (– – –) to the applied magnetic field; (c) 7-nitroxide labeled membranes with their axonal axis parallel (———) and perpendicular (– – –) to the applied magnetic field; (d) 7-nitroxide stearate labeled membranes *after enzyme treatment* with the axonal axis parallel (———) and perpendicular (– – –) to the applied magnetic field.

(*i.e.*, in a qualitative sense; similar changes are observed in the 5- 7-, and 12-nitroxides spectra after enzyme treatment).

Experiments performed with nerve bundles showed that the structural anisotropy produced in the case of the 5-NS-labeled nerve was maximal and, in fact, decreased slightly on enzyme treatment, although at large values of θ, the experimental error is very high (see Table III). The decrease is therefore probably experimental error. The 7-NS showed a large increase in the anisotropy factor on enzyme treatment (θ increased from 1.2 to 1.8) showing that the ordering effect produced by the enzyme is now as high for a nitroxide 7.2 Å from its charged carbonyl group as for that 4.8 Å from its charged carbonyl group. An increase in ordering accompanying enzyme treatment as determined by the nitroxide's mobility, *i.e.*, $\cos^{-1}(\gamma^2)^{1/2}$, is thus confirmed by the data obtained with orientated membranes. These latter observations show that the preferred orientation of the fatty acid chain of the 7-NS before enzyme treatment is perpendicular to the axonal axis, as θ equals 1.2 ($\theta = 1.0$ is the isotropic condition, *i.e.*, the fatty acid chains are orientated equally perpendicular and parallel to the axonal axis). However, the proportion of the fatty acid chains in the preferred orientation (perpendicular) to the axonal axis increases drastically after enzyme treatment.

In Figure 3 are presented the iodoacetamide and the NEM spin-label spectra. The shape of the NEM spin-labeled spectrum is different from that found with iodoacetamide suggesting a different specificity of the reagents for the membrane sulfhydryl groups. Phospholipase A_2 treatment altered drastically the environments of those sulfhydryl groups accessible to the NEM label as shown in Figure 3b, but only slightly those labeled by iodoacetamide (see Figure 3a). The NEM spectrum consists of two components representing a weakly (W) and a strongly (S) immobilized population. After enzyme treatment, the strongly immobilized component appears to grow at the expense of the weakly immobilized component. Treatment of the RBC membranes with lysolecithin (10:100, w/w lysolecithin–membrane lipid) produced similar changes in the NEM spin-label spectrum. Thus both phospholipase A_2 and lysolecithin produced changes opposite to those expected from a detergent (*i.e.*, an increase in the mobile components of the spectrum at the expense of the immobile). Neither treatment produced a vast change in the iodoacetamide spin-label spectrum (see Figure 3c,d).

Polyacrylamide gel electrophoresis employing 1% dodecyl sulfate gels (as described by Lenard, 1970) was performed with the enzyme- and lysolecithin-treated membranes. Gel photographs and densitometer traces are presented in Figure 4. It is seen that after enzyme treatment an increase in the band of app mol wt 76,000 is observed and a slight decrease in that mol wt 108,000. Lysolecithin treatment appears to produce a "similar" increase in the mol wt 76,000 band.

TABLE III: The Structural Anisotropy (θ) as Defined by the Ratio of the Amplitude of A to B (see Figure 2) of a Spin-Labeled Nerve with Its Axonal Axis Parallel to That with Its Axis Perpendicular to the Applied Field (H).

Membrane Prepn and Its Orientation to the Applied Magnetic Field	Spin Label	A/B	θ
Control (perpendicular)	5-NS	0.84 (±0.05)	1.8 (±0.2)
Control (parallel)	5-NS	0.45 (±0.05)	
Treated (perpendicular)	5-NS	1.09 (±0.05)	1.6 (±0.2)
Treated (parallel)	5-NS	0.67 (±0.05)	
Control (perpendicular)	7-NS	0.79 (±0.05)	1.2 (±0.1)
Control (parallel)	7-NS	0.65 (±0.05)	
Treated (perpendicular)	7-NS	1.26 (±0.05)	1.8 (±0.2)
Treated (parallel)	7-NS	0.68 (±0.05)	

The circular dichroism spectra of the treated and control erythrocyte ghosts show (within experimental error) little to no change on enzyme treatment (see Figure 5).

Discussion

The data obtained with the fatty acid spin labels with both the nerve and erythrocyte membrane show that cleavage of the β-ester bonds of the phospholipids has produced a more immobile lipid phase. The effect is very noticeable even with the 12-NS where the nitroxide group is situated 14.5 Å from its charge anchoring head group. This spin label in every membrane so far employed (RBC, mitochondrial, bacterial, and nerve) results in a resonance spectrum typical of a nitroxide in a relatively mobile environment (*i.e.*, describing isotropic motion). The cleavage of the β-ester linkage may therefore allow the phospholipid fatty acid chains to pack together in a more ordered array. In the axonal membrane, this ordering results in a greater number of the fatty acid chains being orientated perpendicular to the axonal axis than in an untreated membrane.

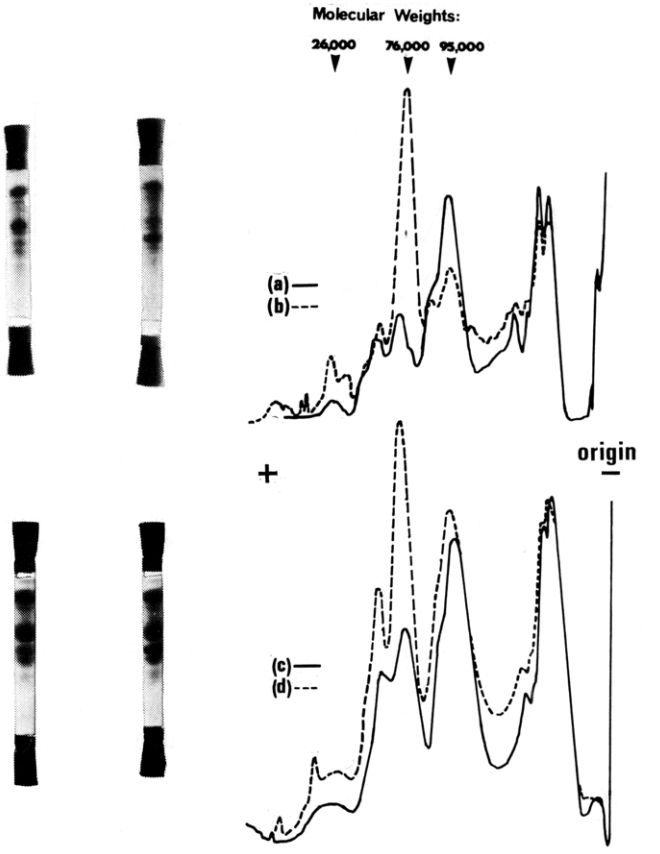


FIGURE 4: Polyacrylamide gel electrophoresis patterns performed according to Lenard (1970) of (a) normal RBC membranes and (b) after phospholipase A_2 treatment. Parts c and d represent the gel patterns obtained with control and lysolecithin treated RBC membrane preparations, respectively. The molecular weight per distance migrated relationship was determined by running gels with hemoglobin (human), BSA, and γ-globulin as molecular weight standards.

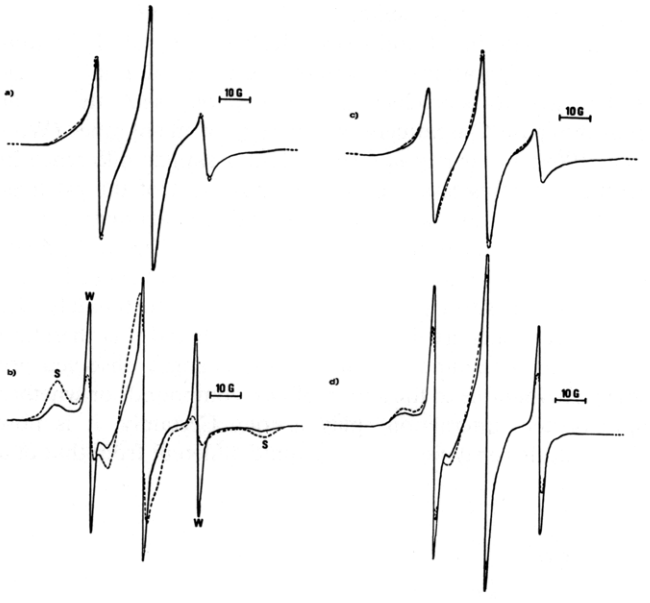


FIGURE 3: The iodoacetamide (a) and *N*-ethylmaleimide (b) spin-labeled RBC membranes before (———) and after (– – –) treatment with phospholipase A_2. Lysolecithin-treated RBC membranes (– – –) as compared to control membranes (———) spin labeled with the iodoacetamide (c) and *N*-ethylmaleimide (d) spin labels.

The protein studies show that very little change has occurred in those proteins containing sulfhydryl groups which are labeled by the iodoacetamide spin label and also that the overall helical composition does not change greatly as measured by circular dichroism. The latter observation is at variance with the results of Gordon *et al.* (1969) who showed a change in shape and size of the circular dichroism spectra of Ehrlich ascites carcinoma cell and RBC plasma membranes after enzyme treatment which ressembled the changes produced by adding lysolecithin (or sodium dodecyl sulfate; Lenard and Singer, 1966). The explanation as to why the two sets of results differ could be (a) that the enzyme concentration employed by Gordon *et al.* was higher, and so perhaps contained enough phospholipid that the addition of enzyme resulted in the production of exogenous lyso compounds, or (b) the sensitivities of the circular dichroism machines. The SS20 attachment to the Jasco-Durrum gives perhaps a five- to ten-times-increased sensitivity. This means that a more dilute membrane suspension can be employed resulting in far less light scattering. The change observed by Gordon *et al.*

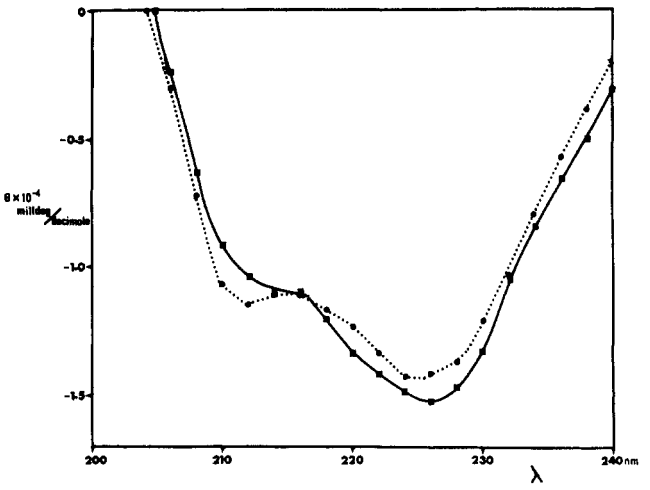


FIGURE 5: Circular dichroism spectra of phospholipase A_2 treated (- - -) and control (———) preparations of RBC membranes.

(1969) may have been due to a change in the light scattering produced by the enzyme-treated membrane suspension. A change in light scattering may affect these results to a lesser degree because lower membrane concentrations could be employed.

The changes in the NEM spin-label spectra produced after phospholipase A_2 treatment show that more sulfhydryl groups have become accessible to the alkylating reagent. The above results, in addition to the compositional data presented in Table I, present strong evidence that no detergent action has occurred during the enzymatic digestion of the membranes. This is because (a) the circular dichroism spectra of RBC membrane show pronounced changes on the addition of detergents (Lenard and Singer, 1966) or lysolecithin (Gordon *et al.*, 1969), but the CD spectra presented here in Figure 5 do not show any such change; (b) it has been shown by Schneider and Smith (1970) that addition of detergent concentrations of lysolecithin (2%) or other detergents (SDS, 0.2%) to red cell ghosts results in the NEM spin-label spectra exhibiting a dramatic change with the *mobile* component of the spectrum growing at the expense of the *immobile* component. The opposite was found to be true after phospholipase A_2 treatment of the RBC membranes. The *immobile* component of the spin-labeled spectrum gains at the expense of the *mobile* component. The iodoacetamide spin-labeled membranes show little change and so it appears that the observed change in the spin-label spectra is somewhat specific. It is also assumed for reasons given elsewhere (Simpkins *et al.*, 1971) that both spin labels are relatively specific for the protein sulfhydryl groups.

The polyacrylamide gel electrophoresis patterns of the phospholipase A_2 treated membranes also show changes in the relative amounts of the protein bands of apparent mol wt 76,000 and 106,000.

Treatment of the membrane with lysolecithin (10% of the lipid weight) results in a similar increase in the mol wt 76,000 band and similar effects on the NEM and iodoacetamide spin-label spectra.

Phospholipase A_2 treatment of the membrane appears to result in some sulfhydryl groups deep in the membrane becoming accessible to spin label. The size of the effect is such that the small amount of the NEM label (maximum 10%) which diffuses into the lipid regions and will therefore reflect the lipid conformation could not result in such a large change. Proteolytic activity in this preparation of phospholipase A_2 appears unlikely as the gel electrophoresis pattern shows no marked decrease in the relative amounts of the high molecular weight bonds and concomittant increases in the lower molecular weight bonds. Also, the action of proteolytic enzymes, such as trypsin, results in vast changes in the gel electrophoresis patterns (Rosenberg and Guidotti, 1969) and a change in the NEM spin-label spectrum opposite to that found after phospholipase A_2 treatment (H. Simpkins, unpublished observations.) The increase in the mol wt 78,000 bond is produced by pretreatment of the membrane by a relatively small amount of lysolecithin as well as enzyme treatment, and may infer that (a) the disaggregation procedure employed prior to running the gels is not complete, or (b) the lysolecithin binds to a protein changing its charge which may change its electrophoretic mobility in this SDS electrophoresis system (Tung and Knight, 1971).

Thus it appears that phospholipase A_2 treatment results in the fatty acid chain of the lipids becoming relatively immobile—an effect that is observed all along the length of the fatty acid chain. From the nerve experiments, it can be said that if bilayer regions of the lipids occur in the axonal membrane, then this "bilayer character" is increased after enzyme treatment. Changes in the protein structure as monitored by the NEM spin label show an increased accessibility of some sulfhydryl groups. Treatment with lysolecithin produces in a qualitative sense a similar perturbation in the protein structure but no change in the lipid conformation (*i.e.*, esr spectrum). Any changes between the circular dichroism curves are small and can be explained by experimental error.

The change produced in the environments of the sulfhydryl groups may be solely a result of binding of lyso compounds with certain proteins or binding in addition to conformational changes produced by alterations in the lipid conformation. No such changes in the protein spin labels spectra are observed after phospholipase C treatment (Simpkins *et al.*, 1971) and the specificities of the two enzymes for the phospholipids of the RBC membrane are known to differ—phospholipase A_2 treatment results in all phospholipids but sphinogmyelin being affected and phospholipase C appears to cleave all but phosphatidylserine (Banjo and Kahlenberg, 1971, manuscript in preparation). A greater decrease of (Na + K)-ATPase activity is produced after phospholipase A_2 than after phospholipase C treatment in a variety of membranes (Wallach, 1969; Fischer *et al.*, 1970), and a similar difference in glucogen binding is apparent after treatment of the rat liver plasma membrane with the two enzymes (Tomasi *et al.*, 1970). It is therefore conceivable that phospholipase A_2 treatment results in lipids proximal to the proteins being affected, which in turn affect the protein structure. Unfortunately, the spin-label results are difficult to quantitate and it is therefore difficult to determine whether all the changes observed are solely attributable to the action of lyso compounds or whether lipid–protein interactions play a role. Certainly, it is now clear that this enzyme's action is quite diffferent from that of a detergent.

References

Augustyn, J. M., and Elliot, W. B. (1969), *Anal. Biochem. 31*, 246.

Bartlett, A. R. (1959), *J. Biol. Chem. 234*, 466.

Blomfield, R., Long, C., Sargent, E. J., and Siddle, A. B. (1966), *Biochem. J. 101*, 44P.

Bloor, W. R. (1928), *J. Biol. Chem. 77*, 53.
Dodge, J. T., Mitchell, C., Hanahan, D. J. (1963), *Arch. Biochem. Biophys. 100*, 119.
Fiehn, W., and Hasselbach, W. (1970), *Eur. J. Biochem. 13*, 510.
Fischer, S., Cellino, M., Zambrano, F., Zampighi, G., Nagel, M. T., Manus, D., and Canessa-Fischer, M. (1970), *Arch. Biochem. Biophys. 138*, 1.
Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957), *J. Biol. Chem. 226*, 497.
Gordon, A. S., Wallach, D. F. H., and Straus, J. H. (1969), *Biochim. Biophys. Acta 183*, 405.
Griffith, O. H., and Waggoner, A. S. (1969), *Accounts Chem. Res. 2*, 17.
Hubbell, W., and McConnell, H. M. (1969a), *Proc. Nat. Acad. Sci. U. S. 63*, 16.
Hubbell, W., and McConnell, H. M. (1969b), *Proc. Nat. Acad. Sci. U. S. 63*, 20.
Hunig, S., and Eckardt, W. (1962), *Chem. Ber. 95*, 2493.
Jones, R. G. (1947), *J. Amer. Chem. Soc. 69*, 2350.
Lenard, J. (1970), *Biochemistry 9*, 1129.
Lenard, J., and Singer, S. J. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1828.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
McConnell, H. M., and McFarland, B. G. (1970), *Quart. Rev. Biophys. 3*, 91.
Mendelson, M. (1969), *J. Cell Biol. 42*, 548.
Rose, H. G., and Oklander, M. (1965), *J. Lipid Res. 6*, 428.
Rosenberg, S. A., and Guidotti, G. (1969), *in* Red Cell Membrane—Structure and Function, Jamieson, G. A., and Greenwatt, T. J., Ed., Philadelphia, Pa., Lippincott, p 93.
Schneider, H., and Smith, I. C. P. (1970), *Biochim. Biophys. Acta 219*, 73.
Simpkins, H., Panko, E., and Tay, S. (1971), *J. Membrane Biol.* (in press).
Tomasi, V., Keretz, S., Ray, T. K., Dunnick, J., and Marinetti, G. V. (1970), *Biochim. Biophys. Acta 24*, 31.
Tung, S., and Knight, C. A. (1971), *Biophys. Biochim. Res. Commun. 42*, 1117.
Waggoner, A. S., Kingsett, T. J., Rottschaeffer, S., Griffith, O. H., and Keith, A. D. (1969), *J. Chem. Phys. Lipids 3*, 97.
Wallach, D. E. H. (1969), *J. Gen. Physiol. 54*, 3s.

Synthesis and Properties of Polyribonucleotides Containing N^2-Methyl- and N^2-Dimethylguanylic Acid in Polyguanylic Acid*

Morio Ikehara† and Masao Hattori

ABSTRACT: Polyribonucleotides containing m^2G and G or m_2^2G and G in various ratios were synthesized from the corresponding nucleoside diphosphates by polynucleotide phosphorylase. While the amount of m_2^2G incorporated into the polynucleotide was proportional to the ratio of m_2^2GDP in the reaction mixture, m^2GDP acted inhibitory to the GDP polymerization and the incorporation was less extensive. The T_m of poly(G,m_2^2G) was 30–50° when the amount of m_2^2G in the polynucleotides <74% and over 80° when m_2^2G >51%. Poly(G,m^2G) and poly(G,m_2^2G) containing a lower amount of methylated guanosines form 1:1 complexes with poly(C), but the polynucleotides containing higher amount of methylated nucleotides could not. These results were supported by mixing curves and CD spectra.

N^2-Methylguanosine (m^2G) and N^2-dimethylguanosine (m_2^2G) are constituents of some tRNAs (Holley *et al.*, 1965; Zachau *et al.*, 1966; Madison *et al.*, 1966; RajBhandary *et al.*, 1967; Takemura *et al.*, 1968; Staehelin *et al.*, 1968). The fact that these modified nucleosides occur at specific positions of tRNA chains suggested some roles of these nucleotides in the function of tRNAs. Pochon and Michelson (1969) studied the physical properties of homopolynucleotides of m^2G and m_2^2G and found that these polynucleotides could not form double-stranded complexes with poly(C).[1] Iwamura *et al.* (1970) have shown strong stacking tendency of a compound containing dimethylguanine. Since Yamazaki *et al.* (1967) developed a method suitable for large-scale synthesis of methylated guanosine, it became easier to obtain larger amounts of substrates needed for polynucleotide synthesis.

In this paper we describe the enzymatic synthesis of analogs of polyguanylic acid (poly(G)) containing m^2G and m_2^2G in various ratios using polynucleotide phosphorylase (Grunberg-Manago *et al.*, 1956). The thermal denaturation of these polynucleotides, their complex formation with poly(C), and their CD properties were studied in order to gain more information about the effect of methylguanosine or dimethylguanosine on the secondary structure of RNA.

Materials and Methods

Synthesis of m^2GDP and m_2^2GDP. m^2GDP and m_2^2GDP were prepared from m^2GMP and m_2^2GMP, respectively (Yamazaki *et al.*, 1968), by the method described by Moffatt and Khorana (1961). m^2GMP or m_2^2GMP (1 mmole) was dissolved in a mixture of water (10 ml) and *tert*-butyl alcohol (10 ml). To the solution was added morpholine (0.54 ml). The mixture was

* From the Faculty of Pharmaceutical Sciences, Osaka University, Osaka, Japan. *Received November 13, 1970.* This work is part IX of Polynucleotides. Part VIII is Ohtsuka *et al.* (1970). This work was partly supported by a grant-in-aid for Scientific Research from the Ministry of Education.

[1] The same results were obtained by us independently (reported at Annual Meeting of Pharmaceutical Society of Japan, 1968).