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PRESENCE AND TURNOVER OF ADENOSINE DIPHOSPHATE RIBOSE IN HUMAN ERYTHROCYTES

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ADP-ribose was detected in human red blood cells (RBC) at 0.45 ± 0.1 μM concentrations. These levels could be estimated after purification of ADP-ribose by means of three sequential HPLC fractionations of RBC extracts. Extraction was performed by sonication of RBC either in trichloroacetic acid, followed by centrifugation, or in carbonate-bicarbonate buffer, pH 10.0, followed by rapid ultrafiltration. Neither procedure of extraction caused artefactual formation of ADP-ribose. Prolonged incubation of intact RBC in isotonic buffer containing labeled orthophosphate resulted in the slow incorporation of radioactivity into ADP-ribose. Identification of the labeled ADP-ribose was confirmed upon incubation of the purified metabolite with nucleotide pyrophosphatase, yielding radioactive 5'-AMP and ribose 5-phosphate, while its exposure to a nonspecific deaminase resulted in the quantitative formation of labeled inosine diphosphate ribose.

NAD^+-dependent mono ADP-ribosylation of specific acceptor proteins is widespread in nature, involving both bacterial toxins and eukaryotic mono ADP-ribosyl transferases (1). The functional roles of these covalent protein modifications, and of reverse de-ADP-ribosylation processes as well, are actively investigated in several cellular systems (2-6), including human red blood cells (7-9). On the contrary, little attention is being devoted to free ADP-ribose, although transferase-independent, acceptor-specific binding of free ADP-ribose to proteins has been observed in some biological systems (10-12).

Recently, a structurally related adenine nucleotide, cyclic ADP-ribose, was identified in sea urchin eggs (13) and reported to derive from NAD^+ in several cellular extracts and in partially purified enzyme preparations (14). Considerable interest on cyclic ADP-ribose arises from its potent calcium-mobilizing activity in cellular and subcellular systems (13),

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Abbreviations: HPLC, high pressure liquid chromatography; Rib 5-P, ribose 5'-phosphate; TEA, tetra-ethylammonium hydroxide; PBS, 20 mM Na phosphate, pH 7.4, containing 146 mM NaCl; TCA, trichloroacetic acid; ADH, alcohol dehydrogenase; Ga 3-P, D-Glyceraldehyde 3-phosphate; Ga 3-PDH, D-Glyceraldehyde 3-phosphate dehydrogenase; 2-PGA, 2-phosphoglyceric acid; Htc, hematocrit; IDP-ribose, inosine diphosphate ribose.

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which makes it functionally comparable to inositol phosphates, although apparently unrelated to them in terms of receptor specificity (15).

Free ADP-ribose has never been described in RBC. The results reported in this paper demonstrate that ADP-ribose is normally present in human RBC and that it is produced in intact erythrocytes. These findings stimulate further investigations on the enzymes involved in the turnover of this metabolite and also on its possible roles in RBC.

MATERIALS AND METHODS

Materials. Nicotinamide [U-^{14}C] adenine dinucleotide (220 mCi/mmol) and [^{32}P]orthophosphate (10 mCi/ml) were from Amity, Milan, Italy. Adenylate [^{32}P]NAD^+ (800 Ci/mmol) was obtained from NEN, Florence, Italy, and separated from contaminant [^{32}P]ADP-ribose through the analytical HPLC described below.

All enzymes were obtained from Sigma Chem. Co. (St Louis, MO): 5'-AMPdeaminase (cat. n. A1907), nucleotide pyrophosphatase (EC 3.6.1.9, cat. n. P 7383), Rib-5P isomerase (EC 5.3.1.6, cat. n. P 9752), phosphoribulokinase (EC 2.7.1.19, cat. n. P 9877), ribulose 1,5-P_2 carboxylase (EC 4.1.1.39, cat. n. R 8000), glyceraldehyde 3-phosphate dehydrogenase/3-phosphoglyceric acid phosphokinase mixed enzymes (EC 1.2.5.12 - EC 2.7.2.3, cat. n. G 8505), alcohol dehydrogenase (EC 1.1.1.1, cat. n. A 3263), NAD^+-glycohydrolase (EC 3.2.2.5, cat. n. N 5263). Antibiotics were from Flow Laboratories, Milan, Italy. All other reagents were of the highest purity grade available.

Preparation of [^{14}C]ADP-ribose. [^{14}C]NAD^+ ($5x10^6$ cpm) was incubated in 0.2 ml PBS with 0.6 IU of NAD^+-glycohydrolase at 37°C. After 90 min the incubation was stopped and deproteinized with TCA (5%, final concentration). TCA was removed with diethylether. Excess diethylether was evaporated under nitrogen and the [^{14}C]ADP-ribose formed was separated from both nicotinamide and the unreacted [^{14}C]NAD^+ by analytical HPLC (see below). The peak corresponding to ADP-ribose was collected and stored at -20°C.

Preparation of [^{32}P]NADH. [^{32}P] NAD^+ ($30x10^6$ cpm, 10.4 nanomoles), was incubated with 0.6 M ethanol and 1 IU of ADH for 15 min at 25° C. Complete reduction of NAD^+ was verified by means of a cycling assay, as reported by Zerez et al. (16).

RBC extracts. Blood samples were obtained from normal volunteers using heparin as anticoagulant. Leukocytes and platelets were removed with a leukocyte removal filter (Sepacell, Asahi Medical Co., Tokyo, Japan). RBC were washed four times with 0.15 M NaCl at 1,400 xg and then submitted to either of the two following procedures of extraction:

a) TCA extraction: aliquots of RBC suspension (84% Htc) were added dropwise, under constant sonication (using a microprobe at a setting of 40 watt for 10 s), to the same volume of ice-cold 20% TCA. The precipitate was immediately removed by centrifugation and the TCA was extracted from the supernatant with diethylether. Residual diethylether was evaporated under a stream of nitrogen.

b) Ultrafiltration: RBC suspensions (84% Htc) containing 20 mM EGTA buffered at pH 7.0, were added to the same volume of carbonate-bicarbonate buffer (20 mM nicotinamide, 20 mM $NaHCO_3$ and 100 mM Na_2CO_3, pH 10.0) under sonication at 0° C. The hemolysate was immediately ultrafiltered at 0° C through a 10 kDa cutoff membrane (43 mm, Amicon YM 10,000, Danvers, MA) and its pH was adjusted to pH 7.5 with CO_2.

HPLC analyses. All reverse phase HPLC analyses were performed on a Hewlett Packard HP1090 instrument equipped with an HP 1040A Diode Array spectrophotometric detector set at 260 nm. Identification of the individual peaks was obtained both by co-elution with known standard compounds and by comparison of UV absorption spectra with those of computer-stored standards. Quantitation of results was achieved with external standards and integration of peak areas was obtained with an HP 79996A analytical workstation.

a) Analytical HPLC. The column was a 5 μm, 100 x 2.1 mm, ODS Hypersil C18 (Hewlett Packard, Milan, Italy). Solvent A was 0.1 M KH_2PO_4 containing 5 mM PIC A reagent (Millipore, Milan, Italy), with the pH adjusted to 6.5 with 5 N KOH. Solvent B was 70% A and 30% methanol, with the pH adjusted to 7.5 with 5 N KOH. The solvent program was a gradient starting at 100% A for 5 min, linearly increasing to 2% B in 3 min, then linearly increasing to

50% B in 24 min. The flow rate was 0.5 ml/min. The threshold of detectability for ADP-ribose and the other adenine nucleotides was 60 picomoles per HPLC analysis.
b) Preparative HPLC (phosphate buffer). The column was a 10 μm, 300 x 7.8 mm, μ-Bondapak (Waters, Millipore). Solvents A and B were the same as for the analytical HPLC. The solvent program was a linear gradient starting at 100% A and linearly increasing to 100% B in 30 min, at a flow rate of 2.5 ml/min. Standard ADP-ribose eluted from the column between the AMP and ADP peaks, at 14.8 min. The threshold of detectability for all adenine nucleotides in this analysis was 380 picomoles.
c) Preparative HPLC (formate buffer). The column was as for the analytical HPLC. Solvent A was 0.01 M formic acid, with the pH adjusted to 4.0 with 1.3 M TEA. Solvent B was 50% A and 50% methanol. The solvent program was a gradient starting at 100% A for 5 min, then linearly increasing to 100% B in 10 min, at a flow rate of 0.5 ml/min. AMP, ADP and ADP-ribose co-eluted at 12 min and were separated from free phosphate (0.8 min).
Purification of ADP-ribose. Extracts obtained either by TCA precipitation or through ultrafiltration of sonicated RBC were supplemented with trace amounts of purified [^{14}C]ADP-ribose as internal standard and submitted to the same procedure of purification. The first step was the preparative HPLC (phosphate buffer). The extract prepared from 1.5 ml packed RBC was injected in three aliquots. The eluent was collected between the AMP and the ADP peaks, dried, re-dissolved in 1/5 of the initial volume and injected in 0.5 ml aliquots into the second preparative HPLC (formate). The desalted adenylic peak eluting at about 12 min was collected, dried, re-dissolved in 0.5 ml water and injected into the analytical HPLC. The purified ADP-ribose eluted in a sharp peak at 7.0 min, well separated from AMP (3.2 min), ADP (9.0 min) and ATP (18.0 min). Both the elution time and the UV absorption spectrum were identical to those of standard ADP-ribose, run separately.
Incorporation of [^{32}P]orthophosphate in native erythrocytes. A 12 ml sterile suspension of RBC (50% Htc) in 0.15 M NaCl buffered with 10 mM Tris-HCl, pH 7.4, was incubated at 37°C in a shaking water bath with 1 mM adenosine, 10 mM glucose (supplemented every 8 h), antibiotics (50 U/ml of Penicillin and 50 μg/ml of Streptomycin) and 1 mCi of [^{32}P]orthophosphate. Aliquots were withdrawn after 2 h (3 ml), 24 h (3 ml) and 32 h (6 ml) and washed five times with 30 vol of 0.15 M NaCl. TCA extracts were obtained as described above. For quantitation of the radioactivity incorporated into ADP and ATP, a 50 μl volume of each RBC extract was submitted to the analytical HPLC. Fractions were collected every 30 s and the radioactivity was determined in a Packard scintillation β-counter. For quantitation of the radioactivity incorporated into ADP-ribose, a 1.5 ml volume of each RBC extract recovered at the various times of incubation was subjected to purification, taking into account the average yield of ADP-ribose after the three step fractionation procedure (50%). After the analytical HPLC the UV absorbing peak corresponding to ADP-ribose was collected in a single fraction and its radioactivity measured.
Digestion of the purified [^{32}P]ADP-ribose with enzymes. The 32 h TCA extract of RBC (1.5 ml), obtained as described above, was supplemented with 150 nanomoles of standard unlabeled ADP-ribose and subjected to the preparative phosphate HPLC. The eluted ADP-ribose was subjected to the preparative formate HPLC. The desalted ADP-ribose was collected, dried, re-dissolved in 0.4 ml of 10 mM formic acid, adjusted to pH 7.0 with 1.3 M TEA and divided into four aliquots of 0.1 ml each, which were kept in ice. One aliquot (zero time) was injected in the analytical HPLC, the fractions collected every 30 s and the radioactivity counted. The three remaining aliquots were incubated in three separate mixtures at 37°C after the following additions, respectively: 1) none; 2) 5 mIU of nucleotide pyrophosphatase and 1 mM $MgCl_2$; 3) 8 mIU of 5'-AMP deaminase. After 30 min incubation, each of the three mixtures was immediately injected into the analytical HPLC column and the eluted radioactivity was measured. HPLC fractions 1-4 from incubation n° 2 were divided into two 0.12 ml aliquots for determination of radioactivity and assay of Rib 5-P (see below).
Assays of NAD^+, NADH and ribose 5-P. NAD^+ and NADH present in the RBC extracts were assayed according to a cycling procedure (16). Rib 5-P was assayed on HPLC fractions by an enzymatic analysis based on its conversion to two molecules of 2-phosphoglyceric acid (2-PGA) and subsequent NADH-dependent reduction of 2-PGA to Ga 3-P. The assay mixture contained in 1 ml: 0.1 M Tris HCl, pH 8.0, 8 mM ATP, 10 mM $MgCl_2$, 0.2 mM NADH, 10 mM $NaHCO_3$, 5 mM GSH, 8 mIU ribulose 1,5-P_2 carboxylase, 10 IU Rib 5-P isomerase, 0.5 IU phosphoribulokinase, 5 IU Ga 3-PDH, and 5 IU 3-phosphoglyceric phosphokinase.

The A^{340} was determined. After addition of sample the mixture was incubated for 30 min at 37°C in a 5% CO_2 atmosphere and the A^{340} was again measured.

RESULTS

Two major causes of artefacts possibly leading to the formation of ADP-ribose during extraction, are: a) some residual NAD^+-glycohydrolase activity acting upon intraerythrocytic NAD^+ ; b) acid-induced degradation of intracellular NADH to ADP-ribose and other breakdown products during the extraction with TCA (4). However, complete inactivation of NAD^+-glycohydrolase was demonstrated by the finding that no hydrolysis whatsoever of added radioactive NAD^+ to yield ADP-ribose was detected with either extraction procedure. Conversely, 6 μM ADP-ribose would be estimated by using the freezing-thawing procedure and delaying the addition of TCA to hemolysates for as little as 10 sec after thawing (not shown). On the other hand, although complete degradation of labeled NADH to ADP-ribose and other products took place in TCA, this did not occur when the addition of TCA to [^{32}P] NADH was performed in the presence of RBC, because of immediate and quantitative oxidation of NADH to NAD^+ (Table 1). This finding confirms earlier observations of a role of hemoglobin in preventing the acid-induced hydrolysis of NADH and NADPH as a result of their conversion to the acid-resistant oxidized forms (17).

The methods of extraction based upon sonication of RBC in TCA or in carbonate-bicarbonate buffer were thus confidently used as adequate systems for exploring the possible presence of endogenous ADP-ribose.

When 1.5 ml erythrocytes was extracted with either procedure, the fractionation of the extracts by three sequential HPLC separations (see "Materials and Methods") revealed a com-

Table I. Effect of hemoglobin during extraction with TCA

Conditions	NAD^+	ADPR	NADH	NADH degradation products
	(percentage of total radioactivity)			
a) NADH in TCA	0	60.0	0	40.0
b) NADH in TCA and sonicated RBC	99.7	0.3	0	0
c) NADH in NaCl	0	0.4	99.6	0

[^{32}P]NADH (10^7 cpm, prepared as described in "Materials and Methods") was added under sonication to each of the following mixtures: a) 100 μl 0.15 M NaCl containing 10 mM nicotinamide, b) 100 μl packed RBC with 10 mM nicotinamide. Immediately after the addition of NADH 100 μl of 20% TCA was added to the mixtures under sonication. Control (c) was prepared by mixing 10^7 cpm [^{32}P]NADH with 200 μl 0.15 M NaCl under sonication. Analytical HPLC was performed on $4x10^6$ cpm of each clear extract, fractions collected every minute and counted. Results are expressed as percentages of the injected radioactivity recovered in the various peaks. The retention times of NADH and of its degradation products were 22 and 24-26 min, respectively. Values are averages of three experiments.

Table II. Labeling of adenine nucleotides in intact RBC incubated in isotonic buffer with [^{32}P]orthophosphate

Incubation (h)	ATP		ADP		ADP-ribose	
	mM	cpm/ nanomole	mM	cpm/ nanomole	μM	cpm/ nanomole
2	1.49	38,600	0.13	20,000	0.32	zero
24	0.69	36,400	0.26	21,900	0.29	3,400
32	0.68	38,700	0.42	24,300	0.33	13,000

Incubations were as described in "Materials and Methods". For each nucleotide the first vertical column indicates intraerythrocytic concentrations and the second one shows specific radioactivities. Results of a representative experiment are shown for the sake of clarity.

pound which had the same UV spectrum and retention time as authentic ADP-ribose. This compound had apparent intraerythrocytic concentrations of 0.45 $\pm$ 0.1 μM. This value was estimated taking into account the relevant yields of radioactivity obtained on the basis of dilution of a known amount of labeled ADP-ribose added to the RBC extracts before the three step purification.

Table II shows the patterns of labeling of ADP-ribose, ADP and ATP in intact RBC exposed to [^{32}P]orthophosphate in isotonic medium. Although the incorporation of radioactivity into ADP and ATP was detectable since 2 h of incubation, appreciable labeling of ADP-ribose was constantly found from 24 h onwards, while it was undetectable at earlier times. In all experiments the intracellular concentrations of ADP-ribose did not show variations from zero time up to 32 h. Structural identification of the adenine compound under study was confirmed by means of enzymes acting upon authentic ADP-ribose. This approach was facilitated by the poor substrate specificity of a commercial 5'-AMP deaminase which proved in preliminary experiments to convert ADP-ribose to IDP-ribose in a quantitative way (not shown). Fig. 1 illustrates the results obtained upon digestion by nucleotide pyrophosphatase and by 5'-AMP deaminase, respectively, of the putative ADP-ribose labeled upon incubation of intact RBC with [^{32}P]orthophosphate and then purified by HPLC. Iden-tification of the radioactive products obtained, i.e. 5'-AMP and Rib 5-P with the pyrophosphatase (Fig. 1C) and IDP-ribose with the deaminase (Fig. 1D), provides unequivocal evidence that the compound under study is ADP-ribose.

DISCUSSION

The results obtained in this study demonstrate that ADP-ribose is normally present in mature human erythrocytes at concentrations that proved to be similar under two different conditions of extraction. Since ADP-ribose was found in deproteinized extracts, it is clearly a free metabolite, i. e. non-covalently bound to proteins.

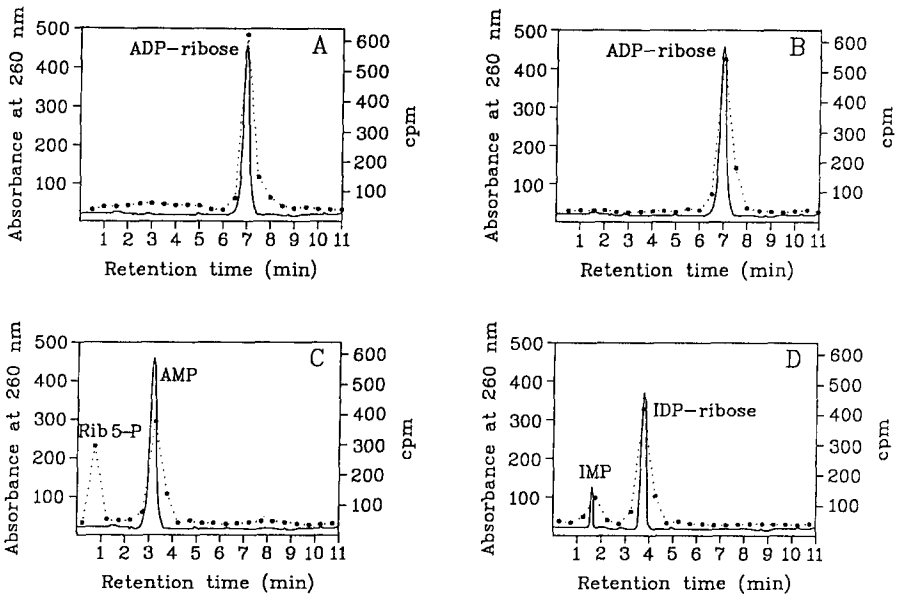

Fig. 1. HPLC analysis of the products of enzymatic digestion of purified [^{32}P]ADP-ribose (see "Materials and Methods"). Zero time (A), 30 min incubation without enzymes (B), 30 min incubation with nucleotide pyrophosphatase (C), 30 min incubation with 5'-AMP deaminase (D). Chromatography profile (———), radioactivity profile (•··•··•··•).

The low levels of ADP-ribose in human RBC could result from: i) its uptake from plasma, similarly to what has been reported for cAMP in human RBC (18), ii) vestigial persistence in RBC since earlier stages in erythropoiesis, iii) formation as a normal metabolite in the mature erythrocytes. The fact that radioactive ADP-ribose is formed in RBC incubated with [^{32}P]orthophosphate indicates that it is produced inside mature erythrocytes, although its turnover is apparently much slower than that of ATP and ADP (Table II).

The presence of free ADP-ribose within human erythrocytes raises a number of questions on, a) its mechanisms of synthesis, b) its turnover and c) its possible roles.

a) Formation of free ADP-ribose within human RBC could result either from cleavage from mono ADP-ribosylated proteins or from synthesis of the free metabolite. A mono ADP-ribosyl transferase and a mono ADP-ribose protein hydrolase have indeed been purified from human RBC (7-8). However, whether and to what extent these reactions take place in native RBC remains to be determined. Synthesis of free ADP-ribose in erythrocytes could also result from a NAD^+-glycohydrolase activity of mono ADP-ribosyl transferase (1). The phosphatidylinositol-anchored ectoenzyme NAD^+-glycohydrolase (19-20) seems not to be involved because of the unavailability of intracellular NAD^+ to this enzyme in RBC.

b) A catabolic pathway of free ADP-ribose is apparently active inside erythrocytes, as its concentration remains constant throughout the 32 h of incubation, despite the production of new labeled molecules (Table II). Preliminary experiments indicate the presence in human RBC hemolysates of a Mg^{++}-dependent dinucleotide pyrophosphatase activity with a rate of hydrolysis of ADP-ribose to 5'-AMP and Rib 5-P of 40 nanomoles/min/ml RBC at 37°C.

c) Whichever the metabolic reactions involved in its turnover, the presence of a steady state of this metabolite in erythrocytes suggests a regulation of the biosynthetic and/or degrada-

tive pathways and consequently a possible functional role for ADP-ribose. Lee et al. have demonstrated that ADP-ribose is a major product of the breakdown of cyclic ADP-ribose, a metabolite with intracellular calcium mobilizing activity in see urchin eggs and other animal tissues (13-14). Although cyclic ADP-ribose has not been described in erythrocytes, it might be metabolically related to ADP-ribose also in human RBC. This possibility, which is currently being investigated, could shed light on the metabolic and functional roles these metabolites may play in human erythrocytes.

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REFERENCES

1. ADP-Ribosylation of proteins (Althaus, F.R.,and Richter, C., Ed.) Springer Verlag Berlin, Heidelberg (1987).
2. Moss, J., and Vaughan, M. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 3621-3624.
3. Richter, C., Winterhalter, K.H., Baumhüter, S., Lötscher, H.R., and Moser, B. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 3188-3192.
4. Payne, D.N., Jacobson, E.L., Moss, J., and Jacobson, M.K. (1985) Biochemistry 24, 7540-7549.
5. Jacobson , M.K., Loflin, P.T., Aboul-Ela, N., Mingmuang, M., Moss, J., Jacobson, E.L. (1990) J. Biol. Chem. 265, 10825-10828.
6. Weis, M., Kass, G.E.N., Orrenius, S., and Moldéus, P. (1992) J. Biol. Chem. 276, 804-809.
7. Tanuma, S., Kawashima, K., and Endo, H. (1988) J. Biol. Chem. 263, 5485-5489.
8. Tanuma, S., and Endo, H. (1990) FEBS Lett. 261, 381-384.
9. Moss, J., and Stanley, S.J. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 4809-4812.
10. Frei, B. and Richter ,C. (1988) Biochemistry 27, 529-535.
11. Hilz, H., Koch, R., Fanick, W., Klapproth, K., and Adamietz, P. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 3929-3933.
12. Richter,C. and Kass, G.E.N. (1991) Chem.Biol.Interactions 77, 1-23.
13. Lee, H., Walseth, T.F., Bratt, G.T., Hayes, R.N., and Clapper, D.L. (1989) J. Biol. Chem. 264, 1608-1615.
14. Rusinko, N., and Lee, H.C. (1989) J. Biol. Chem. 264, 11725-11731.
15. Lee,H.C. (1991) J.Biol.Chem. 266, 2276-2281.
16. Zerez,C., Lee, S., and Tanaka, K. (1987) Anal. Biochem. 164, 367-373.
17. Burch,H.B., Bradley, M.E. and Lowry, O.H. (1967) J. Biol. Chem. 242, 4546-4554.
18. Thomas, E.L., King, L.E., and Morrison, M. (1979) Arch. Biochem. Biophys. 196, 459-464.
19. Pekala, P.H., and Anderson, B.M. (1978) J. Biol. Chem. 253, 7453-7459.
20. Kim,U.H., Rockwood, S.F., Kim, H.R., and Daynes,R.A. (1988) Biochim. Biophys. Acta 965, 76-81.