Rubber Biosynthesis by a *Hevea* Latex Bottom-Fraction Membrane

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ABSTRACT: Washed bottom-fraction particles (WBPs), that is, intact membrane-bound vesicles, prepared from the bottom fraction of centrifuged fresh latex, were shown to be active in in vitro rubber biosynthesis (RB) assays. The RB activity catalyzed by WBP enzymes was confirmed by enzyme parameter criteria. A washed bottom-fraction particle membrane (WBM), prepared from WBPs, was even more active in in vitro RB activity. Mg^{2+} was a cofactor required for RB enzymes, and the cation chelators EDTA and EGTA inhibited the RB process. The temperature had a strong effect on RB stimulation during the heat pretreatment of the WBM before RB assays. A detergent also proved to a strong RB stimulator. The anionic detergent SDS, above the critical micelle concentration, was a strong stimulator of WBM activity. This was not observed with the nonionic detergents Triton X-100 and Tween 20. Various temperature ranges used for WBM preincubation showed a sharp rise in RB activity above 70°C. The temperature required for a sharp rise in RB was lowered substantially in the presence of SDS. It was down to 40°C with the combined preincubation but remained constant with an even higher RB activity. Greater rubber formation at a lower temperature was observed in the presence of SDS. A synergistic effect for RB stimulation appeared during the heat pretreatment of WBM in the presence of SDS. © 2002 Wiley Periodicals, Inc. J Appl Polym Sci 87: 90–96, 2003

Key words: enzymes; rubber; membranes; melting point; micelles

INTRODUCTION

The latex of *Hevea brasiliensis*, a rubber tree, is a complex cytoplasmic system in which rubber particles (RPs) and nonrubber particles are dispersed in an aqueous phase of cytosolic serum. It has long been known that *Hevea* latex contains a large number of nonrubber constituents present with respect to the major RPs. Many of these are dissolved in the aqueous serum of the latex, whereas others are adsorbed onto the RP surface or are suspended as distinct membrane-bound particles. The nonrubber constituents not only are biologically important to the latex metabolism but also affect the physical and chemical properties for the latex colloidal stability. A microscopy study on laticifers of *H. brasiliensis* has shown that the latex is contained in the cytoplasm with three main particulate components (RPs, lutoid particles, and Frey–Wyssling complexes), in addition to typical organelles (i.e., nuclei, mitochondria, and ribosomes). *Hevea* latex can easily be fractionated into three fractions by ultracentrifugation, which provides a top rubber layer, a middle layer of metabolically active aqueous-phase C serum, and a sedimented bottom fraction (BF) of membrane-bound organelles. The BF is mainly composed of membrane-bound organelles called lutoids and some Frey–Wyssling complexes as minor components. Fresh *Hevea* latex is a colloidal mixture of three different particles, the most abundant being RPs, followed by lutoids and the minor Frey–Wyssling complexes, along with the soluble organic and inorganic substances in the aqueous suspension. The structure of *Hevea* latex with its detailed biochemistry has been the subject of a recent, thorough, and extensive review.[1] Studies on rubber biosynthesis (RB) in *Hevea* latex and its control have appeared in several reviews.[2–5] These studies focused on the RP surface as a site for RB investigations. It is still debatable whether RB constitutes elongation steps with preexisting rubber molecules or the formation of new rubber molecules, for which differentiation is difficult to make or verify. However, RB at sites other than the RP surface has received little attention and has rarely been examined. Therefore, the initiation of the synthesis of new rubber molecules and their eventual aggregation to form RPs still remain elusive.

Previous studies on the initiation of RB indicated that washed rubber particle (WRP) surfaces could catalyze the formation of new rubber molecules with FDP, GGDP, and GDP as allylic initiators. However, it

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still remains possible that RB can occur at certain specific sites other than the RP surfaces, as earlier suggested, but has not yet been carefully investigated. Of particular interest is the nonrubber particulate (lutoid particles and Frey–Wyssling complexes) surface in the latex, which might have an important role in the RB process. Unfortunately, little attention has been paid to possible RB activity on the surfaces of these nonrubber particulate components. It seems to be a paradox if we consider the previous numerous studies in which the RP surface was implicated and suggested as the one and only prerequisite site for the in vitro RB process.[6–8] The question then is how and where the original or first RPs are formed in the laticifers and then serve as the required sites for the formation of new rubber molecules by the RPs isolated from latex in those in vitro RB studies with WRPs. No careful systematic study has yet been done or tested for the other alternative sites, except our earlier report on rubber formation by a fresh BF of *Hevea* latex. Therefore, we carried out the experiments discussed in this report to test and examine fresh *Hevea* BF surfaces or membranes for their possible active role and involvement in the RB process.

Among the nonrubber particulate components, lutoid particles and Frey–Wyssling complexes are quite abundant in the latex. Lutoids were first described by Homans et al.[9] as membrane-bound, polydisperse lysosomal vacuoles constituting approximately 20 vol % of fresh latex, and they have been assigned a number of important metabolic functions because of their considerable content in the latex. They are enclosed by a single membrane that is very rich in phosphatidic acids, and this renders them negatively charged vesicles[10] playing a role in latex colloidal stability. Inner lutoids (called B serum) cover a wide ranges of metabolites, proteins, and hydrolytic enzymes that might be considered types of phytolysosomes.[11] The Frey–Wyssling complexes are spherical (4–6 μm in diameter) and are bound with double membranes as composite organelles containing lipid globules, membrane fragment vesicles, and β-carotene.[12,13] Their high carotenoid content suggests that they might contain enzymes for isoprenoid synthesis pathways.[12]These particles could be fractionated into BFs by the centrifugation of fresh latex with the yellow Frey–Wyssling complexes at the upper border of the sedimented lutoids.[1] So far, only a few studies have indicated the related metabolic roles of these particles in isoprenoid and RB pathways.[12,14] The enzyme HMG-CoA reductase, presumably a rate-limiting enzyme in RB,[15] was solubilized and purified from a lutoid membrane.[14] It was shown to be under control by the heat-stable calmodulin as an activator of this enzyme.[16] Isopentenyl diphosphate (IDP) isomerase and prenyl transferase (PT) activities were present in the BF particles and were characterized.[17–19] Recently, we reported active rubber formation by BFs[19] in addition to the presence of IDP isomerase, PT, and rubber transferase in the same BF particles.[18] A kinetic study on ^{14}C-IDP incorporation and an analysis on the appearance of low molecular weight radiolabeled rubber molecules[19] possibly suggested the initiation or new formation of rubber by these intact particles. This article examines further studies on the in vitro RB activities of the membrane derived from the BF particles. A comparison of the effects of the washed bottom-fraction particles (WBPs) and the washed bottom-fraction particle membrane (WBM) on the RB was made first to validate further studies on the RB activities of the particle membrane. Parameters affecting the membrane functions were investigated for the determination of their interactions and effects on the RB process. Among these, the effects of detergents and the heat preincubation of the WBM at different temperatures on the RB activities were extensively characterized. A possible important role of WBM in rubber biogenesis was also examined.

EXPERIMENTAL

Chemicals

[1-^{14}C]IDP (2.04 Gbq mmol^{-1}) was purchased from Amersham. The Tween 20, Triton X-100, and SDS detergents were obtained from Sigma–Aldrich Co. (St. Louis, MO). All other reagents were analytical-grade.

Methods

Preparation of the washed latex BF particles

All operations were carried out at 0–5°C. Freshly tapped latex was collected in an ice-chilled beaker from the regularly tapped rubber trees of an RRIM 600 clone. The latex was fractionated by centrifugation, as described in ref. 14, and this provided a top rubber layer, a middle aqueous latex cytosol (C serum), and the BF. The BF was isolated and washed three times by careful suspension in 5 vol of a 50 m*M* Tris–HCl buffer (pH 7.4) containing 0.9% NaCl (w/v) so that WBPs were obtained. The resulting WBPs were isolated as pellet fractions by centrifugation at 4500*g* for 30 min and were kept in an ice bath until being used for the washed membrane preparation or the in vitro RB assays.

Preparation of the WBP membrane

The WBP sedimented pellet isolated after washing was resuspended in 3 vol of distilled water and stirred for the hypotonic lysis of the WBPs. The resultant membrane was prepared as a cleaned membrane pellet fraction by centrifugation at 5000*g* for 60 min and was washed three times to be obtained as the WBM.

The washing was performed with the same method used for the WBP preparation, and the WBM was kept in an ice bath until it was used.

Heat-preincubation treatments of the WBM

The WBM suspended in a 50 m*M* Tris buffer (pH 7.7) was subjected to heat-preincubation treatments at different temperatures for 30 min. The heat-pretreated WBM was then cooled to room temperature and kept in an ice bath until it was used for further assays of the RB activity under different experimental conditions.

RB incubation and assay conditions

The RB incubation assay mixture contained specified amounts of the WBPs or WBM for experiments on the RB activity, as indicated for the assay conditions in the figure legends. The heat-pretreated WBM was also subjected to the same assay conditions indicated in the Results and Discussion section or similar ones. The incubation mixture was suspended in a 50 m*M* Tris–HCl buffer (pH 7.7) containing 10 m*M* DTT and 5 m*M* $MgCl_2$ and the specified concentrations of [1-^{14}C]IDP (2.04 Gbq mmol^{-1}). In RB assays of the affectors, divalent cation chelators (EDTA and EGTA) or detergents (SDS, Triton X-100, and Tween 20) were also added to the incubation mixtures at specified concentrations. All the incubations were carried out in a shaking water bath at 37°C for 1 or 6 h as indicated.

Assays of radiolabeled rubber for the RB activities

The resultant radiolabeled rubber was precipitated with ethanol from the incubation mixture after incubation with the method described in ref. 19. The rubber was extracted and purified with hexane/toluene (1:1) three times, as similarly reported in ref. 19, so that the purified rubber was obtained. The rubber extracts were concentrated into a small volume by reprecipitation with acetone for three cycles and were finally dried in vacuo. The purified rubber pellets were then dissolved with toluene in scintillation vials for the monitoring of the RB assays under different experimental conditions and for the activity analyses.

RESULTS AND DISCUSSION

Incorporation of ^{14}C-IDP into rubber by the WBPs and WBM

The incorporation of ^{14}C-IDP into rubber by the BF of centrifuged fresh latex was recently reported. Our previous findings on active rubber formation in the BF[19] prompted us to investigate in detail the role of BF particles in the RB process. The earlier study was carried out with unwashed and intact membrane-bound BF particles and the newly formed rubber being analyzed.[19] In this study, the BF particles were thoroughly cleaned and purified as intact WBPs by repeated washings (at least three) with an isotonic buffer. The WBM was prepared by the hypotonic lysis of WBPs and again underwent repeated washings for the RB experiments. A comparison of the effects of the washed particles and the derived washed membrane on the RB activity was made. The results shown in Figure 1 demonstrate the different rates of ^{14}C-IDP incorporation into the rubber by the WBPs and WBM at various ^{14}C-IDP concentrations. The experimental conditions were similar to those used for the WRPs.[7] The findings indicated the active role of latex nonrubber constituents, BF particles, and the derived membrane in rubber formation. The membrane (WBM) activity was about two times greater than that of the WBPs. The higher WBM activity might be attributed to the increased accessibility of substrates for the enzymes. The results suggested not only that the WBM was more active than the WBPs but also that a membrane or membranelike environment was required or necessary for the RB activity. The results (see Fig. 1), combined with a supporting kinetic study (data not shown), indicated that it was an enzyme-catalyzed process being tested and verified in the following experiments on the membrane (WBM) alone.

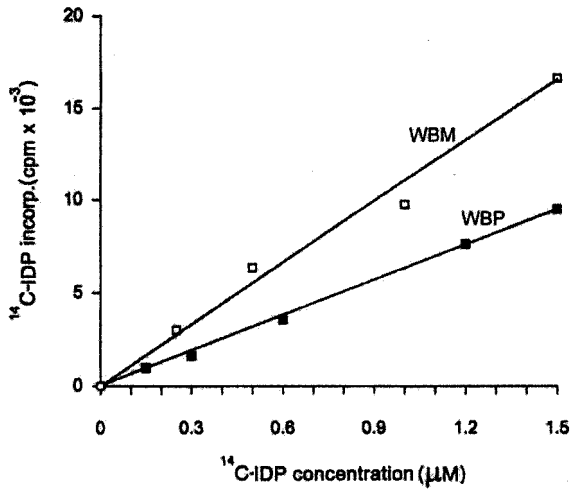

Figure 1 RB by the WBPs and WBM in the presence of various concentrations of [1-^{14}C]IDP. The RB incubation assays contained either a 5-g wet weight of the WBPs or the derived membrane WBM suspended in a 50 m*M* Tris–HCl buffer (pH 7.7), and the reaction was started by the addition of the substrate IDP. Reaction mixtures with 10 m*M* DTT and 5 m*M* $MgCl_2$ were added to [1-^{14}C]IDP at the concentrations indicated. The incubation assay was carried out in a shaking water bath at 37°C for 6 h.

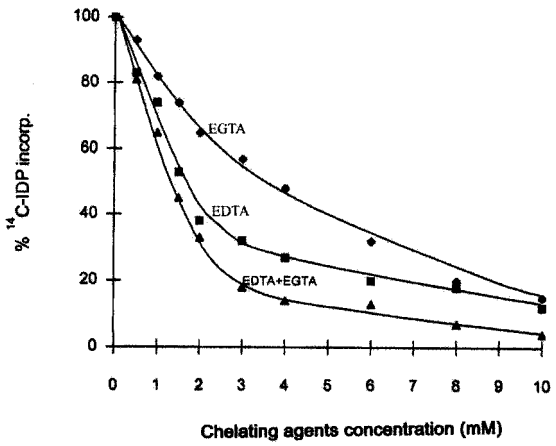

Figure 2 Effect of the chelating agents EDTA and EGTA on the RB activity of the WBM. The conditions for the RB assays were as described in the Methods section. The incorporation of [1-^{14}C]IDP into the rubber was determined for the chelator effects. The incubation mixtures (3 mL) contained 2.5 g of the WBM in a 50 m*M* Tris–HCl buffer (pH 7.7), and EDTA, EGTA, or both were added at the concentrations specified. The incubation was performed in a shaking water bath at 37°C for 1 h.

Effect of the chelators on the RB activity of the washed membrane

Divalent cations were essential cofactors in a WRP study for RB activity.[20] The buffers for both WBP and WBM assays on the RB activity contained Mg^{2+} as reported in earlier studies.[19] In these WBM assays, the addition of chelating agents (EDTA and EGTA) to the incubation showed strong inhibition of rubber formation by the WBM (Fig. 2). A sharp drop in the RB activity was observed with 2 m*M* EDTA (Fig. 2), with almost complete inhibition. The results shown in Figure 2 agree well with the general requirements of divalent cations for the cleavage of phosphate esters by PT enzymes required for RB.[2] When both chelators were present together, the degree of inhibition was not much different than that with EDTA alone. The results indicated that Mg^{2+} was an essential cofactor for the RB enzymes of the WBM, as observed for the WRP assay.[7] The chelator inhibition of the WBM activity on rubber formation suggested that the WBM enzymes might be similar to those reported for the WRP surface.[7] It should also be noted that the slightly higher inhibition level for the combined EDTA and EGTA might suggest a possible bound Ca^{2+} effect on WBM functions, but this remains to be further proved and elucidated.

Effect of the detergents on the RB activity of the washed membrane

The activation of PT and RB activities by detergents has been commonly observed.[7] The effects of different detergents on the WBM for RB activity were determined (Fig. 3). When detergents were included in the RB assay of the WBM, a significant stimulation of ^{14}C-IDP incorporation into rubber was obtained with only SDS, an anionic detergent (Fig. 3). The nonionic detergents, Triton X-100 and Tween 20, showed no effect on RB (Fig. 3). The level of RB stimulation increased with increasing SDS concentrations above the critical micelle concentration (cmc). A threefold activation was detected at 5% SDS, whereas no effect was observed for nonionic detergents. The lipids of the WBM are rich in phosphatidic acid and saturated fatty acyl residues.[10,21] SDS, being anionic in nature, might be miscible or compatible with the negatively charged membrane, having a positive effect on the RB activity. The presence of soluble amphiphiles such as SDS may alter the physical state of WBM lipids and/or induce the formation of newly mixed micelles with increased surface area. These mixed micelles were incorporated with necessary factors and enzymes required for RB. All these events might possibly lead to the increased in vitro RB capacity of the WBM. A refined assay with a very low level, up to 5% SDS, showed biphasic SDS effects (Table I). The RB activity decreased at low SDS concentrations and increased above 1% SDS. The results indicated that RB stimulation by SDS only occurred as the SDS concentration or content was greater than its cmc.

A kinetic study designed to correlate the WBM levels with the rubber formation was examined. It was carried out in the presence and absence of 5% SDS with various WBMs to determine the different degrees of RB capacity. The results (Fig. 4) clearly indicated a strong positive SDS effect on RB stimulation, as shown in the preceding findings (Fig. 3 and Table I). The SDS

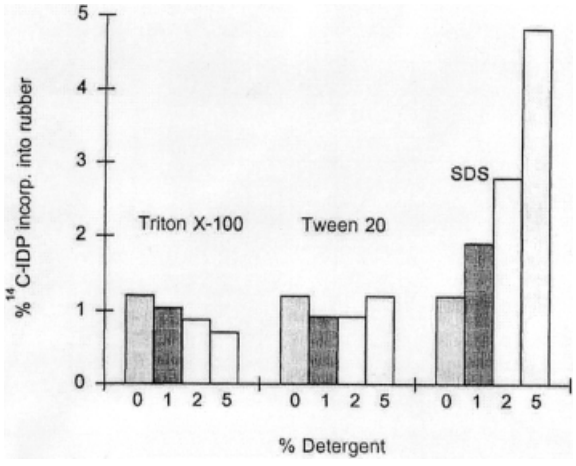

Figure 3 Effect of the detergents on the RB activity of the WBM. The standard assay mixtures were added with different amounts of SDS (0–5%, w/v), Triton X-100, and Tween 20 (0–5%, v/v). The radiolabeled rubbers in the presence of different detergents were characterized at the end of 1 h of incubation. The incubation was performed in a shaking water bath at 37°C for 1 h.

TABLE 1
Effect of Varying the SDS Percentage/RB on Stimulation of (WBM)

SDS (%) in the incubation tubes	[1-^{14}C] IDP incorporation[a]	
	cpm $\times 10^{-3}$	Incorporation[a] (%)
0	2.18	1.70
0.001	1.65	1.26
0.01	1.43	1.10
0.1	2.02	1.16
1	2.48	1.92
2	3.62	2.80
5	6.20	4.84

[a] The data represent the averages of three determinations.

activation effect was even more pronounced at higher contents of the WBM in the incubation. A steady and higher RB stimulation with increasing WBM was clearly seen in contrast to the control incubation without SDS (Fig. 4). A continuously increasing wider gap of the different RB levels was up to 10-fold at higher contents of the WBM in comparison with the smaller difference seen with the lower contents of the WBM. A direct linear relationship between the amounts of the WBM and the rubber formation was, therefore, obtained and was more pronounced in the presence of SDS. SDS could cause the kinetic difference in the WBM enzyme behavior as the control showed an early saturation curve, whereas the SDS sample still showed a continuous rise in the RB activity at corresponding points. This might be assumed to be a cooperative or enhanced effect of SDS on the WBM enzymes, and so the catalytic activity increased accordingly.

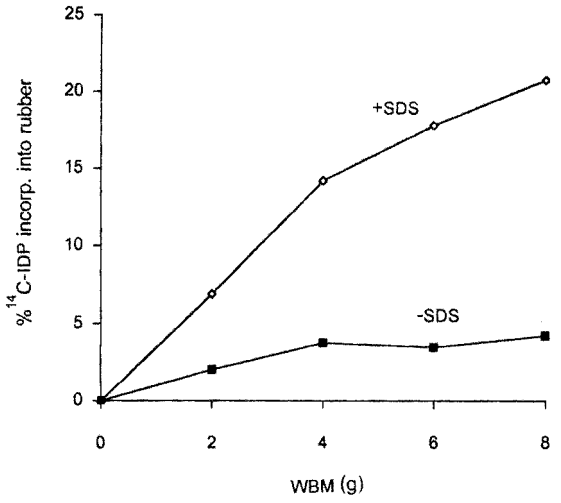

Figure 4 RB activities for different levels of the WBM in the presence or absence of SDS. The standard assay conditions were used, and the reaction mixtures contained various amounts of the WBM as indicated. A comparison was made between the incubation with 5% SDS and the control without SDS. The RB activities were determined for radiolabeled rubber after 1-h incubation assays. The incubation was performed in a shaking water bath at 37°C for 1 h.

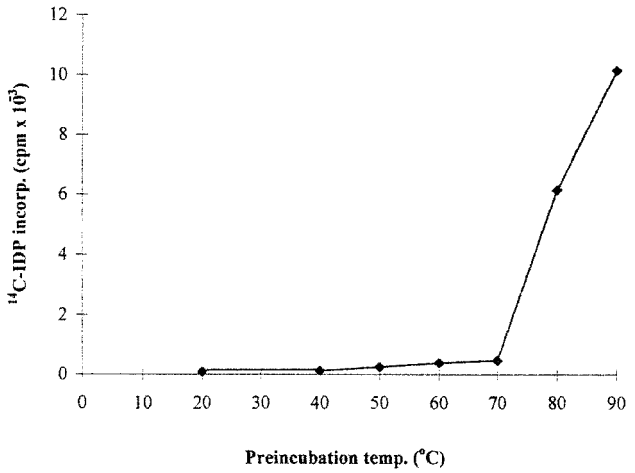

Figure 5 Effect of the heat preincubation on the RB activity of the WBM. The heat-preincubation treatments of the WBM at different temperatures were performed for 30 min, and then it was cooled to room temperature before the standard RB assay. The incubation for the RB activity of the heat-preincubated WBM at different temperatures was carried out in a shaking water bath at 37°C for 6 h.

Effect of the heat preincubation on the RB activity of the washed membrane

The temperature is the other parameter to be considered when we work with membrane functions or the associated enzymes. The heat preincubation of the WBM was determined for the effect on the RB activity. The result was quite astonishing at high temperatures (Fig. 5). In addition to SDS stimulation, the heat preincubation of the WBM could also strongly stimulate the RB activity, as clearly shown in Figure 5. Within the temperature ranges used for WBM preincubation, it was observed that the higher the temperature was, the better the yield was of rubber formation. Below 70°C, very little effect was observed. However, a sharp rise in rubber formation above 70°C was observed. A very large increase occurred at 80°C and even more so at 90°C. This was quite puzzling, as one might question WBM enzyme denaturation. Several repeated assays were performed and were still reproducible; this indicated that the effect was neither erroneous observations nor the trapping of ^{14}C-IDP by the WBM. All the assays and analyses of the results were verified as the resultant rubber was ascertained to be purified rubber by repeated and vigorous purification steps before analysis. Careful analyses of triplicate assays on the incorporation data revealed that the large increase was about 70-fold at 80°C, as shown in Figure 5. The results typically showed that 70°C was the threshold for an abrupt rise in the RB activity. This might reflect the melting temperature of stearic acid, which has been reported to be the most abundant saturated fatty acyl residue in WBM phospholipids.[21] It has been recognized that lipid vesicles or micelles can be in-

duced to form during incubation in the region of their gel at the liquid-crystalline transition temperature.[22] Therefore, the high-temperature preincubation of the WBM might lead to the formation of numerous small micelles and reversed micelles with necessary factors and enzymes to catalyze the rubber formation and, therefore, greatly increase their capacity for the RB process.

The other, more probable explanation for these unexpected but interesting findings is the renaturation of enzymes and other WBM protein components. After the return to the normal conditions after the heat pretreatment for a brief period, it is likely that certain WBM enzymes and proteins might be renatured to their original structures with the RB activity mostly retained. This has been commonly observed for numerous enzymes and proteins, including our report on boiled latex calmodulin[16] with almost full recovery, which might also have occurred in these findings. The reassembling of these RB enzyme systems into the newly formed small micelles and/or reversed micelles might then occur, with something resembling the former membrane configuration. Full recovery is unlikely, and it is expected that a certain loss will occur. However, this might be compensated by the tremendous increase in the micelle surface area far exceeding the certain denaturation loss, which leads to the highly increased RB activity, as shown. These remarkable results for the recovery and stability are indeed quite puzzling and present a challenge for a more detailed study to verify the findings and provide a better understanding of the in vitro RB process.

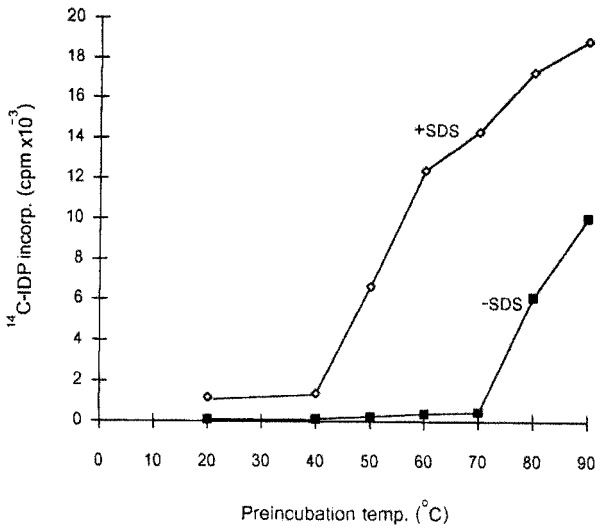

Figure 6 Effect of the heat preincubation and SDS on the RB activity of the WBM. The heat-preincubation treatments of the WBM at different temperatures were performed for 30 min, and then it was cooled to room temperature before the RB assays. The heat-preincubated WBM for the RB assays in a standard mixture either did or did not have 5% SDS. A comparison was made for the temperature effect on the RB activity between the preincubation with SDS and the control without SDS. The radiolabeled rubber was determined for the RB activity after 6-h reaction assays. The incubation was carried out in a shaking water bath at 37°C for 6 h.

Effect of the heat preincubation and the presence of a detergent in the assay on the washed membrane RB activity

Because both the detergent and heat preincubation exerted a strong stimulation on the WBM activities, the combined effect was tested. The aforementioned findings indicated that heat was more effective for RB activation than a detergent, so it was interesting to see how these two parameters would affect the WBM when present together. The result was quite astonishing when the heat preincubation was performed in the presence of 5% SDS (Fig. 6). A sharp rise in rubber formation was reduced, starting from 40°C instead of 70°C without SDS. Higher RB stimulation was observed at all corresponding points with SDS. The phenomena suggested a cooperative and/or synergistic effect of SDS for the heat-preincubated WBM (Fig. 6). Careful analyses of triplicate assays on the activation data revealed that a very large increase was seen at the lower temperatures but that the difference gap was narrowed at the higher temperatures (Fig. 6). Careful comparisons and analyses of the incorporation data in Figure 6 indicated that the large increase with SDS occurred from 50 to 70°C, but the increase was down to a threefold difference at 80°C when the two assays were compared. These results, therefore, indicated that SDS exerted a synergistic effect at an early stage with a lower temperature but then became additive at a later, high-temperature stage as the maximum activation was reached. The same threefold increase was observed with 5% SDS alone (Table I), which then topped the heat activation at 80°C, as shown in Figure 6. Therefore, a synergistic effect was observed at a lower temperature as the lauryl chains in SDS became fluid at the melting temperature (ca. 44°C). Under these conditions, numerous vesicles such as mixed SDS–phospholipid micelles could be induced with the incorporated enzymes to carry out high in vitro RB at a relatively lower temperature. These micelles were, therefore, more effective and suitable to be more active in the RB process. They gave rise to a much higher level of RB activity than the heat-pretreated WBM alone. Other factors might be the anionic character of SDS, which is similar to that of phosphatidic acids, reported to be quite abundant in the WBM,[21] and promotes the formation of new, small micelles and/or reversed micelles with a tremendous increase in the surface area for the highly active RB process being observed.

The results presented here indicate the active role of the fresh latex BF and WBM as possible surfaces or sites for an in vitro RB process. These results also show

the capacity of the membrane-derived lipid micelles for promoting the in vitro RB activity, as shown by the SDS and heat-preincubation effects. Although the BF of centrifuged latex consists mainly of lutoid particles, other cosedimenting particles have also been reported and characterized.[23–25] Yellow-orange Frey–Wyssling complexes are normally found at the upper border of the sedimented BF lutoids.[12] Although a partial contribution by unremovable or unwashable contaminated RPs with the WBM could not totally be ruled out, the heat-preincubated experiments should have removed the heat-coagulated RPs, and the treated WBM would be devoid of active RPs in this in vitro RB study. A high temperature should have inactivated RPs in the heated WBM, so the role of RPs in the RB process might be minimal or ruled out in this membrane study. The idea of lutoids as presumptive sites for rubber biogenesis has been suggested.[24] Our findings might partially support this postulation. However, Frey–Wyssling complexes with high carotenoid contents have also been suggested; they might contain enzymes for polyisoprene synthesis pathways and possibly the RB function.[17] The findings on the membrane activity for RB and the stimulation by parameters that have an effect on membrane functions in this study might be compared to similar effects seen with phospholipid micelles in supporting the polyisoprene synthesis, which has been reported and well characterized.[26] Our results in this study only point out the active role of the BF membrane (WBM) in the RB activity. However, the question still remains concerning which membrane or surface is responsible for the RB activity. The specific identity needs to be elucidated, and the mechanism for its attributed function needs to be clarified. This certainly warrants a further and detailed investigation.

CONCLUSIONS

RB was unequivocally demonstrated in the WBPs, the sedimented membrane-bound particles of centrifuged and fresh *Hevea* latex. The WBM prepared from the WBPs exhibited an even higher RB capacity. The RB activity of the WBM was stimulated by an anionic detergent (SDS), but nonionic detergents (Triton X-100 and Tween 20) had no effect on the WBM activity. The heat-preincubation treatment of the WBM led to a much higher RB stimulation than SDS. The heat-pretreated WBM activity exhibited an even more pronounced RB stimulation in the presence of the SDS detergent. The heat activation of the WBM occurred at a much lower temperature with SDS and with greater RB stimulation. A synergistic effect for the RB stimulation was observed with a combination of the heat pretreatment of WBM and the SDS detergent in the incubation mixtures, and a much greater increase in rubber formation was seen.

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