J. Rubb. Res., **7(1)**, 71–78

On the Possibility of Using ATP Concentration in Latex as an Indicator of High Yield in Hevea brasiliensis.

S. SREELATHA*, SHEELA P. SIMON* AND JAMES JACOB*#

The pool sizes of adenylates, Adenosine 5' tri phosphate (ATP), Adenosine 5' diphosphate (ADP) and Adenosine 5' monophosphate (AMP) in latex, lutoid membrane ATPase activity and cytosolic pH in two high yielding and two low yielding clones were studied during November/December and March/April. There was a strong correlation among concentration of ATP in latex, lutoid membrane ATPase activity and cytosolic pH and all these three parameters were significantly higher in the two high yielding clones than the two low yielding clones during November/December. When measured during summer (March/April), concentration of ATP in latex remained significantly high in the high yielding clones, but lutoid membrane ATPase activity and cytosolic pH gave inconsistent results possibly due to enhanced lutoid disruption, which affected the enzyme activity and cytosolic pH. It is suggested that a large supply of ATP made more energy available for rubber biosynthesis and also it activated the lutoid membrane ATPase activity and thus regulated the cytosolic pH to more optimal levels (less acid) for better conversion of sugar into polyisoprene. The possibility of utilising ATP concentration in latex as an indicator of high yield is discussed.

Key words: adenylates, ATP, ATPase, cytosolic pH, *Hevea brasiliensis*, ADP, AMP, enzyme activity, lutoid, yield, latex

Latex of *Hevea brasiliensis*, the only commercial source of natural rubber is essentially the cytoplasm of specialised cells called laticifers[1]. Regular harvesting of latex causes intense metabolic activity in these cells. This metabolism involves not only rubber biosynthesis but also the reconstitution of other sub-cellular components removed during tapping. A large supply of carbon skeleton and energy (ATP) is required to sustain these processes. Sucrose supply and metabolism[2], particularly respiration and availability of ATP[3] play a major role in these regeneration processes.

Sucrose is converted into mevalonate through the intermediate of acetyl CoA and releases energy in the form of ATP. Mevalonate is then converted into isoprene units that polymerise to give natural rubber with the consumption of significant quantities of ATP[4,5]. ATP and ADP have been shown to be

*Rubber Research Institute of India, Kottayam–686009, Kerala, India
#Corresponding author (e-mail rrii@vsnl.com)

physiological regulators[6] in the whole metabolic pathway leading to rubber biosynthesis The quantity and turnover of adenylate pool have a fundamental importance in latex regeneration and flow and consequently on rubber yield ATP is also the specific substrate for the H^+ pumping ATPase located on the lutoid membrane[8,9] This H^+ ATPase regulates the pH of latex cytosol[10,11] and energises the transport of various solutes inside the lutoids[12] both of which influence rubber biosynthesis

Cytosolic alkalinisation caused by the pumping of protons from the cytosol to the lutoid results in enhanced catabolism of sugars and activates several pH dependent enzymes associated with rubber biosynthesis[13,14] Thus, the adenylate pool size, lutoid membrane ATPase activity and cytosolic pH are key regulators of rubber biosynthesis There is a fairly good understanding of the biochemistry of rubber biosynthesis and the role of adenylates, especially the availability of ATP for rubber production Although the possibility of characterising clones based on laticiferous functioning has been suggested by Jacob *et al* [15], there has been no conscious attempt to apply this fundamental knowledge for any tangible practical advantage in crop improvement such as developing a high yielding clone The present experiment is an attempt in this direction with the specific objective of examining the relationship between the concentrations of ATP, ADP and AMP in the latex, ATPase activity of the lutoid membrane and C-serum pH with latex yield

MATERIALS AND METHODS

Plant Material

The study was carried out in four clones RRII 105 and RRIM 600 (representing high yielders), HP 20 and RRII 38 (representing low yielders) planted during 1989 at the Rubber Research Institute of India, Kottayam Eight trees per clone having comparable girth and yield were selected The trees were in the second year of tapping under ½S d/2 6d/7 tapping system (half spiral alternate daily tapping system, three tapping days per week) Total latex yield, concentrations of adenylates (ATP, ADP and AMP) in the latex, lutoid ATPase activity and C-serum pH were measured during the peak-yielding (November/December) and low yielding summer (March/April) seasons Latex was collected 5 minutes after tapping so that the initial destabilised latex was avoided The sample was taken for about 6–8 minutes directly from the tree into sample vials kept in ice Sampling was done on four tapping days in the first season and three tapping days in the second season

Estimation of Adenine Nucleotides

Adenine nucleotides in latex were determined according to Amalou and co-workers[16] Fresh latex samples (~1 0 gm) were extracted with 0 5 N Trichloroacetic acid (TCA) The TCA was then removed by repeated extractions with cold ether and the residual ether was evaporated The samples were then neutralised with 0 1 N potassium hydroxide (KOH) and the volume was made up to 10 mL with 30 mM Hepes (N- [2-Hydroxy ethyl] piperazine-N'-[2-ethane sulphonic acid]) –Tris (Tris hydroxy methyl aminomethane) buffer pH 7 4 and used for measuring adenylates Coagulated rubber was rinsed twice with TCA and then dried at 80°C to determine the dry rubber content of the latex sample

ATP was quantified by the bioluminescence method using the bioluminescent kit (FL-AA 89 H 9803, Sigma Chemical Company, USA),

which contained luciferin-luciferase complex. To 100 µL TCA extract added 100 µL assay mix from the kit and ATP was measured using a Luminometer (Stratec electronic GmbH, Brikenfeld, Germany). ADP and AMP were assayed after being phosphorylated to ATP using pyruvate kinase and adenylate kinase[17]. Adenine nucleotide concentrations were expressed in µmoles and the adenylate energy charge (AEC) was calculated according to Atkinson[6] as given below:

$$AEC = \frac{[ATP] + 1/2\,[ADP]}{[ATP] + [ADP] + [AMP]}$$

Determination of C-serum pH and Lutoid Membrane ATPase Activity

The cold latex samples were centrifuged at 59 000 g for 45 min at 4°C to separate C-serum, lutoids and the rubber particles. The rubber cream was discarded and the clear C-serum was directly used for pH measurement. The bottom fraction consisting of lutoids were collected and suspended in 5 volumes of 50 mM Hepes- (N-[2-Hydroxy ethyl] piperazine-N'-[2-ethane sulphonic acid]) Mes- (2- [N-Morpholino] ethanesulfonic acid) Tris (pH 7.0), 300 mM mannitol buffer. The crude lutoid fraction was washed three times with the same buffer. It was then centrifuged at 35000 g for 10 min at 4°C.

ATPase assay was performed in 2.5 mL assay buffer[18] (50 mM Hepes -Mes-Tris pH 7.0, 300 mM mannitol, 5 mM $MgSO_4$, 0.1 mM ammonium molybdate) with 10% lutoid. The reaction was started by the addition of 100 µL 5 mM ATP at pH 7.0. The incubation time was 10 min at 26°C under continuous stirring. Then adding 200 µL ice cooled 0.5 mM TCA stopped enzymatic hydrolysis of ATP. The inorganic phosphorus (Pi) released during the hydrolysis of ATP by ATPase was measured spectrophotometrically[19].

Protein content of this washed lutoid fraction was estimated by the method of Lowry *et al.*[20] with BSA as standard. Samples were precipitated with 10% TCA, centrifuged at 7500 g for 5 min at room temperature and solubilised in 0.1N NaOH. ATPase activity was expressed as µmole Pi liberated/min/mg protein.

Determination of Bursting Index

Bursting index of latex was determined according to Ribaillier[21].

RESULTS AND DISCUSSION

Out of the four clones used in the present investigation, both RRII 105 and RRIM 600 had significantly large latex yield ($p<0.001$) compared to the two low yielding clones *viz.* HP 20 and RRII 38 during November/December (*Table 1*) and summer (data not shown). Among the three adenylates whose concentrations were determined in the latex, ATP and ADP were significantly more in both the high yielding clones than either of the low yielding clones at $p<0.02$ and $p<0.05$, respectively (*Table 1*). The adenylate energy charge was significantly large in the two high yielding clones ($p<0.05$), but AMP and ATP/ADP did not show any significant difference between the high yielding and low yielding clones (*Table 1*). The C-serum pH and ATPase activity were significantly high in the high yielding clones compared to the low yielding clones ($p<0.01$, *Table 1*).

All the data collected during November/ December were pooled together and regressed to determine the correlations among the

concentrations of adenylates, AEC, ATPase, C-serum pH and latex yield (*Table 2*) Highly significant positive correlations were observed among ATP, ATPase and C-serum pH and all of them showed significant positive correlations with latex yield ATP had a significantly large positive correlation with AEC while AMP had a nearly equally strong negative correlation with AEC

Significant positive correlations between yield and C-serum pH have been reported earlier[22,23] A large C-serum pH enhances rubber biosynthesis pathway, because several key rate-limiting enzymes *e g* alkaline invertase in glycolysis[7], involved in these processes are activated by slightly higher pH It is suggested that the high rates of activities of ATPase present on the lutoid membrane led to efficient pumping of protons from the cytosol into the lumen of the lutoids and thus increased the pH of the C-serum favouring glycolysis and rubber biosynthesis[2,18,24] It has been shown that ATPase activity and cytosol alkalinisation increased with the application of ethephon, which lead to increased latex production[17] Stimulating the trees with ethephon application has shown an increase in total adenine nucleotides in latex and cytosolic alkalinisation[16]

A large supply of ATP makes rubber biosynthesis more efficient not only by making more energy available for the conversion of mevalonate into polyisoprenes but also by raising the cytosolic pH through ATPase activity as inferred from the positive correlations existing among them (*Table 2*) Thus ATP is a very important regulator in rubber biosynthesis through its direct effect on the metabolic pathway (supplying energy) and indirect effect mediated through ATPase activity and thus modulating cytosolic pH to favourable levels When virgin trees were freshly opened and tapping progressed, the rate of respiration in the bark gradually increased[3] and the tapping also induce a sink demand for sucrose, which is used as a substrate for respiration as well as rubber biosynthesis In TPD affected trees inhibition in latex biosynthesis was associated with significantly smaller concentrations of ATP in the latex[25] It has been reported that ethylene activated carbohydrate metabolism leading to increased rubber production[2] and Ethrel® application led to increased adenine pool size which lead to high latex yield[16]

When the summer results were analysed, ATP concentration in the latex was significantly more ($p<0.05$) in the two high yielding clones than the two low yielding clones as seen during November/December, but ATPase and C-serum pH did not show any significant difference (data not shown) unlike the highly significant difference observed during November/December This indicates that ATP concentration in latex is a more reliable indicator of yield than lutoid ATPase activity or C-serum pH The inconsistency in the results of lutoid ATPase and C-serum pH between the two seasons may be associated with the likely instability of the lutoids during summer It may be noted that lutoids are less stable during summer[26] and their bursting will release large amounts of protons into the cytosol and thus alters the C-serum pH Disrupted lutoid membrane may affect the activity of ATPase that is present on this membrane Although the latex samples were collected from the trees into vials that were pre-cooled in ice and the samples were kept on ice throughout the measurements, lutoids were found to be less stable in summer than in November/December (*Figure 1*) This showed that lutoid instability during summer is independent of the ambient temperature and likely to be dependent on some intrinsic tree factor related to summer drought However, when analysed during November/December

TABLE 1. CLONAL VARIATIONS IN YIELD, POOL SIZES OF ADENYLATES IN LATEX, LUTOID ATPase ACTIVITY AND C-SERUM pH IN *HEVEA BRASILIENSIS* LATEX DURING PEAK YIELDING PERIOD (NOVEMBER–DECEMBER)

Category	Clones	Yield (mL/tree/ tap)	ATP µM	ADP µM	AMP µM	ATP/ ADP	AEC	ATPase µmole Pi/min/mg protein	C-pH
High yielding	RRII 105	163	244.53	330.98	126.87	0.73	0.57	5.06	6.95
	RRIM 600	152	224.86	279.84	102.86	0.87	0.61	4.08	6.85
Low yielding	HP 20	66	174.79	150.1	286.72	1.16	0.41	2.31	6.56
	RRII 38	75	129.82	198.51	135.52	0.65	0.49	2.65	6.51
		***	**	*	ns	ns	*	***	***

*CD $p \leq 0.05$; **$p \leq 0.02$; ***$p \leq 0.01$; ns: not significant

TABLE 2. INTER RELATIONSHIP BETWEEN YIELD, POOL SIZES OF ADENINE NUCLEOTIDES, ATPase ACTIVITY AND C-SERUM pH IN *HEVEA BRASILIENSIS* LATEX DURING NOVEMBER/DECEMBER

Parameter	ATP	ADP	AMP	ATP/ADP	AEC	ATPase	C-pH
Yield	0.63**	0.13	0.01	0.33	0.48*	0.60**	0.60**
ATP		0.34	0.11	0.45*	0.64**	0.51*	0.38
ADP			0.29	−0.59**	−0.03	0.14	0.22
AMP				−0.16	−0.63**	0.13	−0.17
ATP/ADP					0.50*	0.35	0.12
AEC						0.30	0.37
ATPase							0.62**

*Significant at $p \leq 0.05$; **Significant at $p \leq 0.01$

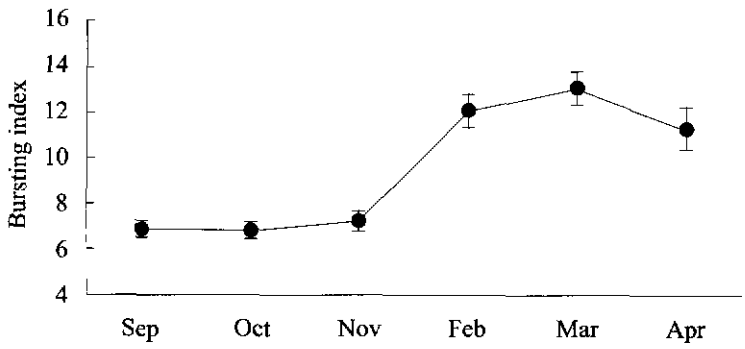

Figure 1. Variations in bursting index of clone RRII 105 during the peak yielding post-monsoon (September-November) and low yielding summer (February-April) seasons.

(which is practically a stress free period) lutoid ATPase activity, C-serum pH and ATP concentration in the latex were good indicators of high yield Given the central regulatory role of ATP in rubber biosynthesis and its seasonal insensitivity as discussed above, it is suggested that ATP concentration in latex is a good candidate marker for high yield If the present findings obtained from mature trees can be established in immature trees and extended to more number of clones, ATP concentration in latex can be potentially used as a screening criterion for early prediction of high yield Work is already in progress in this direction

ACKNOWLEDGEMENT

The authors gratefully acknowledge the help rendered by Sri Ramesh B Nair, Assistant Director (Statistics) for carrying out statistical analysis of the data

Date of receipt October 2003
Date of acceptance January 2004

REFERENCES

1 D'AUZAC, J AND JACOB, J L (1989) The Composition of Latex from *Hevea brasiliensis* as a Laticiferous Cytoplasm *Physiology of Rubber Tree Latex The Laticiferous Cell and Latex—A Model of Cytoplasm* (*D'Auzac J* et al *eds*), pp 59–88 Florida CRC Press

2 TUPY, J AND PRIMOT, L (1976) Control of Carbohydrate Metabolism by Ethylene in Latex Vessels of *Hevea brasiliensis* Mull Arg in Relation to Rubber Production *Biologia Plantarum*, **18**, 988–994

3 ANNAMALAINATHAN, K KRISHNAKUMAR, R AND JAMES JACOB (2001) Tapping Induced Changes in Respiratory Metabolism, ATP Production and Reactive Oxygen Species Scavenging in *Hevea J Rubb Res* , **4(4)**, 245–254

4 KECKWICK, R G O (1989) The Formation of Isoprenoids in *Hevea* latex *Physiology of Rubber Tree Latex The Laticiferous Cell and Latex—A Model of Cytoplasm* (*D'Auzac J* et al *eds*), pp 145–165 Florida CRC Press

5 JACOB, J L AND PREVOT, J C (1992) Metabolism of the Laticiferous System and its Biochemical Regulation *Natural Rubber Biology Cultivation and Technology* (*Sethuraj M R and Mathew N M eds*), pp 116–136 New York El Sevier

6 ATKINSON, D E (1977) Cellular Energy Metabolism and its Regulation New York Academic Press, 293 pp

7 JACOB, J L , PREVOT, J C , LACROTTE, R , GOHET, E , CLEMENT, A , GALLOIS, R , JOET, T , PUJADE-RENAUD, V AND D'AUZAC, J (1997) The Biological Mechanism Controlling *Hevea brasiliensis* Rubber Yield *IRRDB Symposium on Natural Rubber (Hevea brasiliensis) V2 Physiology and Exploitation and Crop Protection and Planting Methods, 14–15 October Ho Chi Minh City Vietnam*, pp 1–11

8 D'AUZAC, J (1977) ATPase Membranaire des Vacuoles Lysosomales Les Lutoids du Latex d' *Hevea brasiliensis Phytochemistry*, **16**, 1881–1889

9 CHRETIN, H (1982) The Proton Gradient Across the Vacuo-lysosomal Membrane of Lutoids from the Latex of *Hevea brasiliensis* 1 Further Evidence for a Proton Translocating ATPase on the Vacuo-lysosomal Membrane of Intact Lutoids *J Membrane Biology*, **65**,174–184

10 CHESTIN, H, GIDROL, X, MARIN, B, JACOB, J L AND D'AUZAC, J (1984) Role of the Lutoidic Tonoplast in the Control of the Cytosolic Homeostasis within the Laticiferous Cells of *Hevea brasiliensis* *Zeitschrift Fuer Pflanzenphysiologie*, **114 S**, 269–277

11 MARIN, B, MARTIN LANZA, M AND KOMOR, E (1981) The Protonmotive Difference Across the Vacuo-lysosomal Membrane of *Hevea brasiliensis* (Rubber Tree) and its Modification by a Membrane bound Adenosine Triphosphatase *Biochemical J* **198** 365–372

12 MARIN B, CRETIN, H AND D'AUZAC, J (1982) Energisation of Solute Transport and Accumulation at the Tonoplast in *Hevea* Latex *Physiologia Vegetale*, **20**, 333–346

13 JACOB, J L, PREVOT, J C AND D'AUZAC, J (1982) Physiological Activators of Invertase from *Hevea brasiliensis* Latex *Phytochemistry*, **21**, 851–858

14 TUPY, J (1973) The Level and Distribution Pattern of Sucrose along the Trunk of *Hevea brasiliensis* Mull Arg As Affected by the Sink Region Induced by Latex Tapping *Physiologie Vegetale*, **11**, 1–8

15 JACOB, J L, PREVOT, J C, LACROTTE, R, CLEMENT, A, SERRES, E AND GOHET, H (1995) Clonal Typology of Laticifer Functioning in *Hevea brasiliensis* *Plantation Recherche Developpment*, **2(5)**, 43–49

16 AMALOU, Z, BANGRATZ, J AND CHRESTIN, H (1992) Ethrel (Ethylene releasor) Induced Increases in the Adenylate Pool and Transtonoplast pH within *Hevea* Latex Cells *Plant Physiology*, **98**, 1270–1276

17 HANSEN, O H AND KARL, D M (1978) Biomass and Adenylate Energy Charge Determination in Microbial Cell Extracts and Environmental Samples *Methods in Enzymology*, **LV111**, 73–85

18 GIDROL, X CHRESTIN, H, MOUNOURY, G AND D' AUZAC, J (1988) Early Activation by Ethylene of the Tonoplast H^+ Pumping ATPase in the Latex from *Hevea brasiliensis* *Plant Physiology*, **86**, 899–903

19 TAUSSKY H H AND SHORR E (1953) A micro-colorimetric Method for the Determination of Inorganic Phosphorus *J Biological Chemistry*, **202**, 675–685

20 LOWRY, O H, ROSEBROUGH, N J, FARR, A L AND RANDALL, R J (1951) Protein Measurement with Folin Ciacalteau Reagent *J Biological Chemistry*, **200**, 193–265

21 RIBAILLIER, D (1968) Action *in vitro* de Certain Ions Mineraux et Composes Organiques sur la Stabilite des Lutoides du Latex d' *Hevea* *Revue Generale des Caoutchoucs Plastiques*, **45(12)**, 1395–1398

22 ESCHBACH, J M, ROUSSEL, D, VAN DE SYPE, H AND JACOB, J L (1984) Relationship between Yield and Clonal Physiological Characteristics of Latex from *Hevea brasiliensis* *Physiologie Vegetale*, **22**, 295

23 CRETIN, H, JACOB, J L, PREVOT, J C AND D'AUZAC, J (1980) Le pH du Latex, Son Influence sur la Production et les Elements de sa Regulation *Revue Generale des Caoutchoucs Plastiques*, **603**,111

24 JACOB, J L PREVOT, J C, AND KECK-WICK, R G O (1989) The Metabolism of the Laticiferous Cells of *Hevea brasiliensis* *Physiology of Rubber Tree Latex The Laticiferous Cell and Latex—A Model of Cytoplasm* (*D Auzac J et al eds*) pp 101–144 Florida CRC Press

25 KRISHNAKUMAR, R, ANNAMALAINATHAN, K, SHEELA, P SIMON AND JAMES JACOB (2001) Tapping Panel Dryness Syndrome in *Hevea* Increases Dark Respiration but not ATP Status *Indian J Nat Rubb Res*, **14(1)**,14–19

26 PREMAKUMARI, D, SHERIEF, M AND SETHURAJ, M R (1980) Lutoid Stability and Rubber Particle Stability as Factors Influencing Yield during Drought in Rubber *J Plantation Crops*, **8**, 43–47