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Nucleic Acids in Latex and Production of Rubber in Hevea Brasiliensis

J. TUPY

Institut des Recherches sur le Caoutchouc au Cambodge, Kompong-Cham, Cambodia

Some modifications of current methods for the analysis of nucleic acids in latex are described.

In regard to nucleic acids, Hevea *latex contains both ribosomal RNA and soluble RNA; apparently it has also DNA and messenger RNA. The latter was tentatively identified according to its position on MAK chromatograms and by its rapid labelling with* P^{32}.

The latex possesses some capacity for RNA synthesis in vitro. *The rate of this synthesis as well as the total amount of nucleic acids in the latex are directly related to the production of rubber. Some evidence was obtained that this relation concerns essentially ribosomal RNA and supposed messenger RNA, while the amount of soluble RNA seems rather to decrease with latex of high regenerating activity.*

The presence of ribosomes in *Hevea* latex (McMULLEN, 1959 and 1962) is to be considered as an important indication of its protein synthesising ability.

However, in spite of the presence of RNA in latices of many other plant species, no significant incorporation of labelled amino acids into their latex proteins *in vitro* has been found (MEISSNER, 1966).

But in view of the very low ability of these plants to regenerate their latex, this finding cannot be generalised. Quite another situation with respect to protein synthesis and general metabolic activity is to be supposed in latices from regularly tapped *Hevea* trees.

To examine the importance of nucleic acids in *Hevea* latex their relation to the latex-producing potentials of clonal trees was tested. An attempt was also made to demonstrate in the latex the presence of messenger and transfer RNA which are necessary for the functioning of latex ribosomes.

MATERIALS AND METHODS

Where not otherwise stated, trees about eighteen years old of clone PR 107 tapped on 2S/2.d/3–d/4 have been used for the experiments. The latex, refrigerated already during tapping, was submitted to analysis about 15 min after collection. Only the first fraction of 10 – 15 ml of latex was used. The aqueous phase was isolated from treated latex (see below) by centrifugation at about 18 000 *g* for 30 min under refrigeration.

Quantitative Determination of Nucleic Acids

The pH of the latex was made up to 7.0 – 7.5 with NaOH and the nucleic acids were extracted with a solution of 0.02 M TRIS buffer, 0.02 M EDTA, 0.1 M NaCl, 3.6% SDS (sodium dodecylsulphate), pH 8.0. To 1.8 ml of latex 2.5 ml of this solution was added and the mixture was vigorously shaken for about 3 min. By centrifugation at 18 000 *g* for 30 min only two phases, an almost clear aqueous phase and the white fraction were obtained. Aliquots of the aqueous phase were precipitated with 2 vol. of alcohol containing 0.56 *N* $HClO_4$. The fine precipitate was three times washed with 50% alcohol — 0.2 *N* $HClO_4$. The nucleic acids were then extracted with 0.5 *N* $HClO_4$ for 30 min at 70° and measured at 260 nm. The optical characteristics of the final extract approximated those of nucleic acids:

$$\lambda \text{ max} = 260 \text{ nm}, \ \lambda \text{ min} = 235 \text{ nm}$$
$$\text{and } E_{max}/E_{min} = 2.0$$

Many modifications of the current methods of nucleic acid determination were tried, in-

cluding, *e.g.*, perchloric acid precipitation of the extract or hot NaCl extraction of the pellet. The modification described above seems to be most suitable for routine analyses.

The details of these studies will be published elsewhere.

Isolation and Fractionation of Nucleic Acids

To about 40 ml of latex 30 ml of 0.02 M TRIS pH 8.0 containing 2% of SDS was added and the mixture was vigorously shaken for 10 min. The aqueous phase obtained by centrifugation at 18 000 *g* was mixed with 0.1 vol. of 10% NaCl and precipitated with 2 vol. of chilled alcohol. After some hours of standing at about −5° the precipitate was separated by centrifugation and extracted twice with 10 ml of 1% SDS in 0.01 M TRIS buffer pH 7.5. The extract was twice agitated with phenol saturated with TRIS–SDS solution. The phenol phases were re-extracted twice with TRIS–SDS and the combined aqueous phases were precipitated by 0.1 vol. of 20% CH_3COOK and 2 vol. of alcohol. After overnight standing at about −5° the precipitate was dissolved in the starting 0.05 M phosphate buffer, pH 6.7 containing NaCl and loaded on a methylated albumin—kieselguhr (MAK) column.

The MAK column was prepared and used as described by MANDEL AND HERSHEY (1960) in the modification without the middle layer. The linear gradient was made from 250 ml each of 0.3 M and 1.2 M NaCl. The optical transmission of the eluate from the column was recorded at 254 nm with an LKB Uvicord II. For quantitative comparison the extinctions of individual fractions at 260 nm were determined using a Beckman DU spectrophotometer.

P^{32} Incorporation

Incubations were carried out at a concentration of about 15 μC of P^{32} sodium phosphate per ml of latex and for 1 h at 30°.

Extraction of nucleic acids and precipitation of the extracts was the same as in the above method of quantitative determination. The pellet washed with 50% alcohol—0.2 *N* $HClO_4$ was further purified by two washings with alcohol–ether (3 : 1) at 50°. The nucleic acids were then extracted with boiling 10% NaCl at pH about 7.2, twice for 30 min and precipitated with CH_3COOK and alcohol. For complete removal of low molecular P^{32} labelled contaminants, the final preparation was filtered on a Sephadex G-25 column (SEBRING AND SALZMAN, 1964). In the high molecular fraction optical density at 260 nm and radioactivity were determined.

For determination of P^{32} incorporation in individual nucleic acid fractions the above isolation and chromatography on an MAK column were used. Each 6 ml fraction of the effluent was mixed with 0.75 mg of carrier albumin and the nucleic acids were then precipitated in 5% TCA. The precipitates separated by centrifugation were dissolved in 0.4 *N* NH_4OH and transferred on to aluminium planchets. All measurements were carried out in a GM counter with an efficiency of about 10^4 c.p.m. per μC.

RESULTS

Nucleic acid Content in Latex and Productivity

If nucleic acids in the latex were functional, their concentration would be different with different rates of latex formation, that is, would differ with the productivity of corresponding trees.

In many experiments with different clonal material a positive relation between the productivity and nucleic acid content in the latex was found. In these experiments the comparison of high yielders and low yielders was always made between trees of the same clone, age and trunk thickness.

According to our experiments most of the latex nucleic acids is present in the serum fraction obtained by centrifugation of the fresh untreated latex at 18 000 *g*. To enable comparison of latices with different T.S., the amount of nucleic acids was expressed per unit of the latex water, this being calculated as the difference between the fresh and dry weight of the latex. But the accuracy of such a comparison of different latices is somewhat affected by the fact, that the proportion of the serum and the total latex water is not constant and that some nucleic acids are closely associated

with the rubber particles (McMULLEN, 1962).

Therefore, to demonstrate the relation between the content of nucleic acids in the latex and productivity, the data obtained with latices of different T.S. are presented *(Table 1* and *Figure 1)*. High production is as a rule associated with lower T.S. of the latex, but even with the pairs of trees, rather exceptional, where this is inverse, the relation between nucleic acids and productivity is still clearly apparent. As the tapping intensity of 2S/2.d/3–d/4 to which the experimental trees were adapted was not changed during the experiment, the general drop in T.S. presented in *Figure 1* is to be considered as a result of changes in weather conditions.

TABLE 1. COMPARISON OF NUCLEIC ACIDS CONTENT IN LATEX OF HIGH- AND LOW-YIELDING TREES[a]

Pairs of high (H) and low (L) yielders	1st		2nd		3rd	
	H	L	H	L	H	L
Total solids content, %	40.70	37.98	43.90	46.40	37.18	42.21
Rubber per S/2 and tapping, g	33.6	7.4	33.2	16.0	25.5	10.9
NA per ml of latex water, µg	317	236	313	262	328	253
Significance of differences.	Highly significant ($P<0.01$)		Highly significant ($P<0.01$)		Highly significant ($P<0.01$)	

[a] Means of nine determinations (latex was taken from nine successive tappings on 2S/2.d/3–d/4 of each pair of trees).

Further evidence of the importance of nucleic acids in the latex for latex regeneration was obtained by following the changes in their level in the latex in the course of intensive tapping *(Figure 2)*. The results for the first day show that the period of about 8 hours is not sufficient for renewal of nucleic acids exported by the morning tapping in trees adapted to the tapping frequency d/3–d/4. Later on the difference in the nucleic acid content between the morning and afternoon latices decreases and their level in latex serum increases progressively. On the other hand on subsequent reduction of the tapping frequency the increase of nucleic acid concentration stops and some days later a decrease sets in.

This experiment also confirms the positive relation between the nucleic acid content in the latex and the rate of latex regeneration.

Fractionation of Latex Nucleic Acids

As an approach to the question whether all the types of RNA at present known to participate in protein synthesis occur in the latex, the MAK column fractionation of nucleic acids has been used. The overall MAK pattern of latex nucleic acids closely resembles that obtained frequently for other plant tissues.

The two typical chromatograms presented show seven distinct fractions according to u.v. absorption *(Figure 3)*. In analogy with chromatographic patterns from other plant material (*e.g.*, KEY AND INGLE, 1964; CHROBOCZEK AND CHERRY, 1966) the individual fractions were tentatively identified as soluble RNA (1 and 2), DNA (3), light and heavy ribosomal RNA (4 and 5 respectively) and messenger RNA (6 and 7).

The fractions 6 and 7 are often masked by the most abundant fraction 5 as is the case on chromatogram B. The small amount of supposed DNA does not surprise considering the very small number of nuclei in the latex (DICKENSON, 1965).

The relative amount of individual fractions varies considerably with different latices. This seems to be related to the latex-producing potential of the trees as is shown in *Table 2*.

For the reasons that not all seven fractions are sufficiently separated and that m-RNA could not be well detected according to the u.v. absorption record with latices from low-yielding trees, only four fractions were considered in this comparative study. The selected high-yielding trees were further stimulated with 2,4-D to highly differentiate their production from that of low yielders. Both samples of latices obtained by mixing of first 10 ml fractions from six high-yielding and six low-yielding trees were analysed simultaneously on the same day.

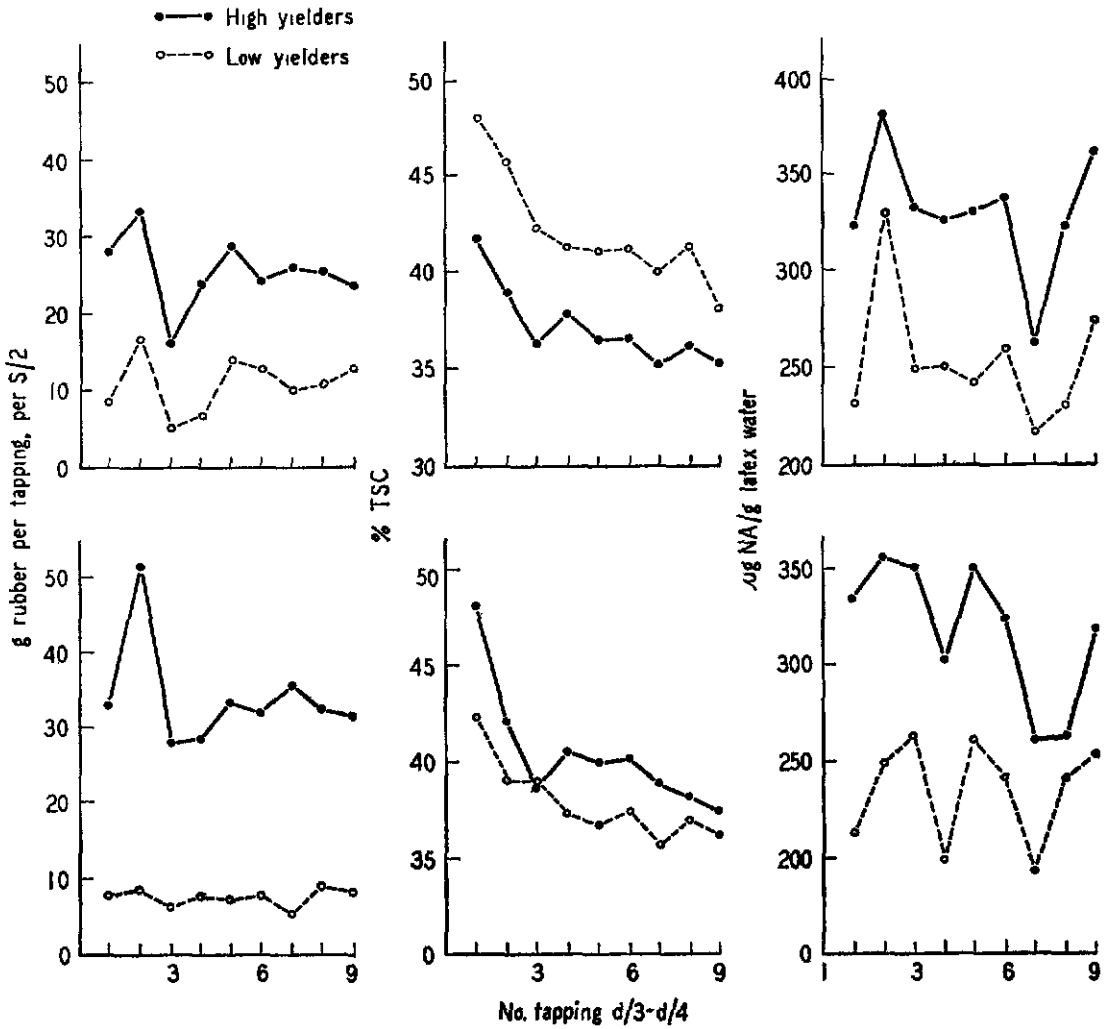

Figure 1. The production of rubber and the content of nucleic acids in the latex. The upper set of graphs gives the individual measurements on the third pair of trees for which results are summarised in Table 1. *The lower set of graphs refers to the first pair of trees mentioned in* Table 1.

The results *(Table 2)* of each of six analyses effected in January show that high production of rubber is related above all to predominance of light ribosomal RNA (lr-RNA) and particularly of the fractions containing heavy ribosomal RNA (hr-RNA) and m-RNA. On the other hand a low level of s-RNA is found in the latex of high-yielding trees.

P^{32} Incorporation into Nucleic Acids

The latex of high-yielding trees contains more nucleic acids in spite of the larger amount of latex exported by tapping. This means that the rate of nucleic acid synthesis in latex vessels must increase with production of rubber.

To test this question we have assayed the ability of the latex to incorporate P^{32} into RNA

and the relation of the incorporating capacity to latex regeneration.

As the procedure of final purification of nucleic acid preparations on a Sephadex G-25 column seems to be efficient enough for separation of all low-molecular contaminants, one can attribute the radioactivity found to the P^{32} incorporation into nucleic acids.

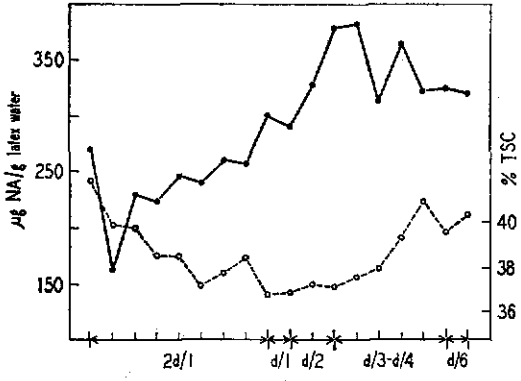

Figure 2. Influence of intensive tapping on nucleic acid content of the latex. Trees of clone GT 1 were adapted before the experiment to the tapping system S/2+2S/3.d/3–d/4. In the experiment, the trees were tapped twice daily (2d/1) at 7 a.m. and 4 p.m. (first to ninth tappings), then daily (d/1) (tenth tapping), then alternate daily (d/2) (eleventh and twelfth tappings), then third daily with weekly day of rest (d/3–d/4) (thirteenth to seventeenth tappings), and finally sixth daily (d/6) (eighteenth tapping). Figures for nucleic acid content (●—●—●) and T.S. (○—○—○) are means of analyses on four trees.

The latex thus seems to be capable of synthesising RNA *in vitro*. The rate of this synthesis according to specific activity of nucleic acid preparations is related to the production of rubber *(Table 3)*. The different rates of RNA synthesis with latices of high- and low-yielding trees further exclude the possibility of explaining the P^{32} incorporation by microbial activity.

Because of great differences in both ribosomal RNA (r-RNA) and supposed m-RNA content, the rate of P^{32} incorporation into these fractions in latices from differently yielding trees has been compared *(Figure 4)*. The same plant material as in the experiment presented by *Table 2* was used. The results obtained confirm the above findings. RNA is synthesised by latex *in vitro*, this synthesis being strongly related to the rate of latex regeneration. The highest specific activity was found in fractions 6 and 7, which further indicates their possible identity with m-RNA. With latex from low-yielding trees their presence was detected by P^{32} labelling only.

DISCUSSION

In addition to the fact that latex contains ribosomes (McMULLEN, 1959 and 1962), the demonstration of the presence of both classes of r-RNA, of s-RNA and presumably of m-RNA strongly suggests that proteins are synthesised in the latex. The proportion of individual fractions in the latex is very similar to that in other

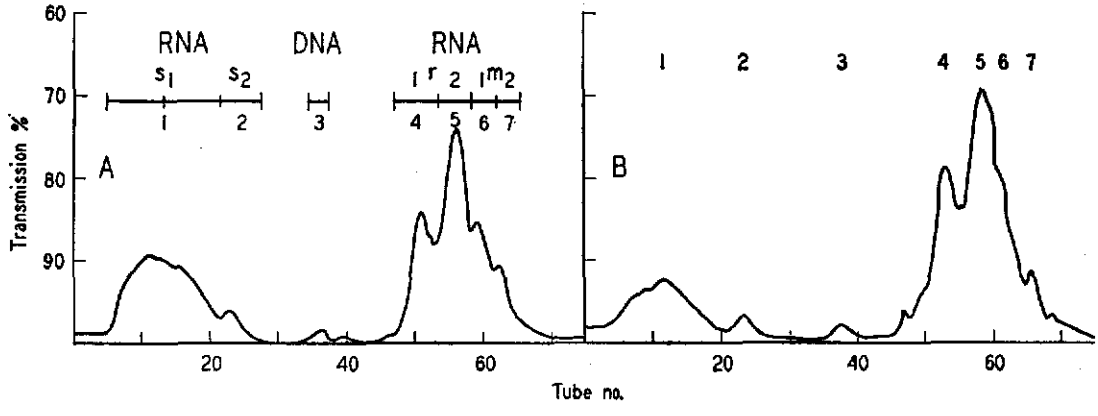

Figure 3. MAK separation of nucleic acids isolated from latex.

TABLE 2. PROPORTION OF NUCLEIC ACIDS FRACTIONS IN LATICES FROM HIGH-YIELDING (H) AND LOW-YIELDING (L) TREES

	$S_1 + S_2 - RNA$[b]				DNA[c]				lr-RNA[d]				hr-RNA, m-RNA[e]				Total			
	H		L		H		L		H		L		H		L		H		L	
	ODU[a]	%	ODU	%	ODU	%	ODU	%	ODU	%	ODU	%	ODU	%	ODU	%	ODU	%	ODU	%
1	2.4	7.2	8.8	35.9	1.2	3.6	0.5	2.0	9.7	29.0	5.5	22.4	20.2	60.3	9.7	39.6	33.5	100.1	24.5	99.9
2	7.2	17.3	11.8	45.9	0.5	1.2	0.2	0.8	12.1	29.1	5.6	21.8	21.8	52.4	8.1	31.5	41.6	100.0	25.7	100.0
3	5.0	14.6	10.0	35.0	1.1	3.2	0.4	1.4	11.6	34.0	6.5	22.7	16.4	48.1	11.7	40.9	34.1	99.9	28.6	100.0
4	2.2	5.9	9.9	42.1	1.1	3.0	0.3	1.3	11.1	30.0	5.3	22.6	22.6	61.1	8.0	34.0	37.0	100.0	23.5	100.0
5	6.1	15.6	11.8	37.3	0.6	1.5	0.1	0.4	12.1	31.0	6.4	20.3	20.2	51.8	13.3	42.1	39.0	99.9	31.6	100.1
6	10.0	22.3	15.2	42.7	1.2	2.7	0.4	1.1	11.7	26.1	7.6	21.3	22.0	49.0	12.4	34.8	44.9	100.1	35.6	99.9
Mean	5.5	13.8	11.3	39.8	1.0	2.5	0.3	1.2	11.4	29.9	6.2	21.9	20.5	53.8	10.5	37.2	38.4	100.0	28.3	100.0

[a] ODU (optical density unit) = $E_{260} \times$ ml
[b] soluble RNA; corresponding with peaks 1 and 2 in *Figure 3.*
[c] supposed DNA; corresponding with peak 3 in *Figure 3.*
[d] light ribosomal RNA; corresponding with peak 4 in *Figure 3.*
[e] heavy ribosomal RNA plus supposed messenger RNA; corresponding with peaks 5, 6 and 7 in *Figure 3.*

TABLE 3. P^{32} INCORPORATION INTO NUCLEIC ACIDS *IN VITRO*: COMPARISON OF LATICES FROM HIGH- AND LOW-YIELDING TREES

Pairs of high (H) and low (L) yielders	1st		2nd		3rd	
	H	L	H	L	H	L
Rubber per tapping, g	31.4	17.4	25.6	11.2	30.0	20.8
Specific activity c.p.m./1000ODU	216	94	296	93	216	120

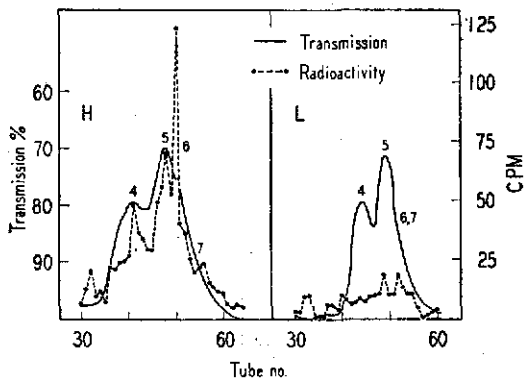

Figure 4. Part of elution profiles from MAK column of P^{32} labelled nucleic acids from latices of high-yielding (H) and low-yielding (L) trees.

plant material, the r-RNA constituting most of the total RNA.

The m-RNA was tentatively identified according to its position on the MAK chromatogram. With respect to the hr-RNA it is identically localised as m-RNA or DNA-like RNA with important messenger properties from, *e.g.*, peanut cotyledons (CHROBOCZEK AND CHERRY, 1965; 1966) corn mesocotyl or soybean tissues (KEY AND INGLE, 1964; LIN, KEY AND BRACKER, 1966). One of the basic properties of m-RNA is a high rate of turnover. In fact the supposed m-RNA is the most rapidly synthesised fraction of latex RNA. But the capacity to form hybrids with a homologous DNA fraction, as well as to direct amino acid incorporation into protein has still to be tested.

In view of the demonstrated RNA synthesis the presence of functional DNA in the latex is to be supposed. Moreover a nucleic acid fraction at the position of DNA in MAK elution profiles was found.

The latex DNA may originate from nuclei which are occasionally expelled from vessels with the latex (DICKENSON, 1965). But it can be also considered as some kind of non-nuclear cytoplasmic DNA, the existence of which is reported in a variety of organisms (*e.g.*, YOO AND JENSEN, 1966).

The regeneration of the latex can, to a certain extent, be compared with the increase of the cytoplasm during growth which is known to require active protein synthesis depending on the concentration of RNA in the cell. In bacteria the RNA synthesis is a simple linear function of growth rate (for literature see NEIDHARDT, 1964). In plants, evidence was obtained that RNA and protein synthesis are essential processes for cell elongation and in the mechanism of growth-promoting auxin action (NOODEN AND THIMANN, 1963, 1965 and 1966; KEY, 1964; KEY AND SHANNON, 1964; HAMILTON *et al.*, 1965).

In analogy with this it was found that in latex the RNA concentration and synthesis are adjusted to the rate of latex regeneration. But the positive relation between the rate of latex formation and RNA level concerns r-RNA and supposed m-RNA only, while the s-RNA is related rather inversely to the production of latex.

This further resembles the situation in bacteria, where also r-RNA only but not s-RNA is directly related to the rate of growth (NEIDHARDT, 1964). For an explanation one may consider the suggestion of BAGULEY AND RALPH (1966) on the origin of s-RNA, according to which s-RNA may be formed by partial degradation of ribosomal precursor RNA which is not rapidly incorporated into ribosomes.

The apparent co-ordination of RNA synthesis with latex regenerating capacity may be

associated with the genetic regulation of synthesis of enzymes concerned with latex formation. Such a suggestion fits the available evidence that rubber production is directly related to the enzymatic activity of the latex *in vitro* (D'AUZAC AND PUJARNISCLE, 1963; CHAI KIM CHUN *et al.*, 1969).

In the current models of genetic regulation of protein synthesis, the primary role is attributed to genetic transcription restricted to the nucleus. But evidence has been obtained that the regulation of synthesis of specific enzymes can also take place in enucleated cells (SPENCER AND HARRIS, 1964). If the latex expelled from vessels lacks nuclei or if their content is too low, this could be considered as an explanation of some RNA synthesising capacity of the latex *in vitro*.

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DISCUSSION

Chairman: Mr. G. F. J. Moir

Dr. W.L. Resing contributed the following additional data on behalf of Dr. Tupy.

Increased production of latex, stimulated by application of 2,4-D and intensive tapping, was accompanied by an increase in the level of both ribosomal and messenger RNA. The latex of high-yielding trees contained larger amounts of nucleic acid despite the greater loss of latex by tapping. The rate of nucleic acid synthesis in the latex vessels must therefore increase with the enhanced production of rubber.

Latex had some capacity to incorporate P^{32} into nucleic acids *in vitro*. This incorporation was related to the rate of latex regeneration in a comparison of latex from low- and high-yielding trees of the same clone. Stimulation by 2,4-D increased the rate of incorporation of P^{32} into the nucleic acids of latex *in vivo*, when P^{32} was injected into bark.