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# The Occurence of Ribonucleic Acid in the Lutoïd Fraction (Lysosomal Compartment) from *Hevea brasiliensis* Künth. (Müll.-Arg.) Latex

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*Summary.* The lutoïds from *Hevea brasiliensis* latex represent a polydisperse lysosomal compartment. They contain RNA which is resistant to RNase in conditions which maintain the integrity of the lutoïds but is hydrolyzed when these organelles are destabilized. This RNA appears to be a structural component of the lutoïds.

## Introduction

Fresh latex from *Hevea brasiliensis* (Künth) Müll.-Arg. contains specific organelles of the lacticiferous cells: the lutoïds and the Frey-Wyssling particles. The lutoïds are single-membrane vesicles containing hydrolytic enzymes: they represent a lysosomal compartment (Pujarniscle, 1969) with some vacuolar properties (Ribaillier *et al.*, 1971). The Frey-Wyssling particles are complex structures bounded by a typical double unit-membrane envelope and they are very rich in carotenoïds containing lipid globules: they are considered to be degraded chloroplasts or chromoplasts (Dickenson, 1969; Coupé *et al.*, 1972). Other organelles, *e.g.* nucleus and mitochondria, have not been seen in the latex (Pujarniscle, 1969; Cockbain and Southorn, 1962).

After centrifugation for 20 min at $47000 \times g$, the *Hevea* latex is separated into three main fractions (Moir, 1959): the liquid middle fraction, referred to as latex serum, contains most of the soluble substances normally present in plant cells; the bottom fraction (sediment) is formed by lutoïds although it also includes varying amounts of rubber and Frey-Wyssling particles; the top fraction is constituted of broken Frey-Wyssling particles and the rubber phase.

McMullen (1959) was the first to demonstrate the presence of RNA in the latex. He showed that some RNA was associated with readily sedimentable material and was liberated from it by treatment with deoxycholate. This particulate RNA had the characteristics of ribosomal bodies. Tupy (1969), Coupé and d'Auzac (1972) and Marin *et al.* (1974) confirmed the occurrence of RNA

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in the latex and showed that this RNA was present essentially in the serum fraction.

In this paper, we confirm the presence of RNA in *Hevea* latex, and show that it is also present in the lutoïd fraction. As this fraction is morphologically heterogeneous, it is further purified and the presence of RNA in the lutoïd organelle is investigated.

## Material and Methods

*Latex Collection. Hevea brasiliensis* latex is collected as described before (Pujarniscle, 1969). Where not otherwise stated, different trees of the clones $GT_1$ or $TJ_1$ are tapped for these experiments. The tapping panel is carefully cleaned with 80% alcohol and dried before tapping. The first fraction of 10–50 ml is discarded because it is very rich in bacteria (Taysum, 1961).

*Preparation of Crude Lutoïd Fraction.* All experiments are performed on the lutoïd fraction prepared according to Pujarniscle (1969). Latex diluted with 1–3 volumes of Tris-mannitol buffer (10 mM KCl, 10 mM $MgCl_2$, 300 mM mannitol, 100 mM Tris-HCl adjusted to pH 7.3) is centrifuged at $39000 \times g$ for 10–20 min at 4°. The pellet, resuspended in 5–20 ml of the same buffer, is the crude lutoïd fraction and the supernatant constitutes the crude serum fraction. The agglomerated poly-isoprenoïd particles which collect above the supernatant form the rubber fraction.

Addition of Neutral red to the collected latex modifies lutoïd density and the crude lutoïd fraction was also prepared from such latex. Neutral red is added in a ratio of 20 mg per 100 ml of latex.

*Sucrose Density-gradient Centrifugation.* The crude lutoïd fraction is washed once with Tris-mannitol buffer. Density-gradient centrifugation is carried out according to Pujarniscle (1969). An 8-ml sample is layered upon a continuous 40–70% or 0–50% sucrose gradient made up in the same buffer. It is centrifuged in a Spinco SW-25.1 rotor at $56000 \times g$ for 15 min (for lutoïd charged with Neutral red) or 120 min (for untreated lutoïds). The tube is punctured and 16–18 fractions are collected.

*Washing Procedure to Remove Contamination from the Lutoïd Fraction.* The crude lutoïd fraction is washed 5 times with the Tris-mannitol buffer described above, with or without EDTA (50 mM). After each washing, the sediment obtained by centrifugation of the lutoïd suspension at $34800 \times g$ for 10 min at 4° is resuspended in some ml of the same buffer, and an aliquot is removed for the different estimations.

*Destabilization Methods for the Lutoïd Fraction.* A twice-washed bottom (lutoïd) fraction suspended in Tris-mannitol buffer is treated by different destabilizing factors:

1. Mechanical shock—breaking of the organelles of the bottom fraction with a Waring blendor at full speed for 1–2 min at 4°.

2. Osmotic shock—resuspension of the fraction with some ml of buffer as used for preparation of the lutoïd fraction but without mannitol.

3. Addition of digitonine (final concentration of 0.5% w/v) (Coupé *et al.*, 1972) or Triton X-100 (final concentration of 0.1–1% w/v) to the resuspension buffer.

After treatment, the bottom fraction is centrifuged at $39000 \times g$ for 15 min at 4°. RNA content, acid phosphatase and polyphenol-oxidase activities of the sediment are estimated as described previously (Pujarniscle, 1969).

*Digestion of Lutoïd RNA with Pancreatic Ribonuclease.* Intact or destabilized preparations of crude lutoïd pellet washed twice with Tris-mannitol buffer are incubated at 37° in the reaction mixture (0.3 M mannitol, 0.1 M sodium acetate, pH 5.0) with 100 µg/ml of pancreatic ribonuclease (Sigma; RNase A type II-A). Digestion is stopped by the addition of 2 volumes of an alcoholic solution of 0.56 N perchloric acid containing 1% (w/v) sodium dodecyl sulphate (SDS). The suspension is centrifuged at $39000 \times g$ for 15 min and residual sedimentable RNA is determined in the pellet by the method of Tupy (1969).

*Analytical Methods.* The different fractions are assayed for protein and RNA content and for enzyme activities.

Proteins are estimated by the procedure of Lowry *et al.* (1951).

RNA content is determined according to Tupy (1969). Nucleic acids are extracted with a solution of NaCl-Tris, pH 8.0 (20 mM EDTA, 100 mM NaCl, 20 mM Tris-HCl with 3.6% w/v SDS). Of this solution 2.5 ml is added to 1.8 ml of fraction and the mixture is vigourously shaken for about 3 min. Centrifugation at $18000 \times g$ for 30 min at 4° separates a white pellet from an almost colorless supernatant. Aliquots of the supernatant are precipited with 2 ml of alcohol containing 0.56 N $HClO_4$. The fine precipitate is washed 3 times with a 50% (v/v) alcoholic solution containing 0.2 N $HClO_4$. Nucleic acids are then extracted with 0.5 N $HClO_4$ for 30 min at 70° and measured at 260 nm.

The activities of acid phosphatase and polyphenol oxidase, which are enzymatic markers of lutoïds and Frey-Wyssling particles, respectively, are assayed by the method of Pujarniscle (1969) for the first activity and the method of Lance (1963) for the second one.

Acid-phosphatase activity measured without detergent and under isosmotic conditions is referred to as free activity. The latency is determined from estimation of free and total acid-phosphatase activities according to Pujarniscle (1969).

*Bacterial Contamination.* Bacterial contamination is measured by plating diluted samples of lutoïd fractions on nutrient agar (Difco Bacto Citrate Agar); bacterial colonies are counted after 48–72 h incubation at 30° and the number of colonies is multiplied by 10 to account for bacteria that would not grown on this medium. Bacterial RNA content is estimated assuming the mean dry weight of $10^6$ bacteria to be $2.5 \times 10^{-7}$ g and the mean bacterial RNA content to be 20% of the dry weight.

## Results

### *Presence of RNA in the Lutoïd Fraction*

Distribution of acid-phosphatase and polyphenol-oxidase activities and of RNA content between the rubber, lutoïd and serum fractions is shown in Table 1. Most of the nucleic acids of the latex are present in the serum fraction obtained by centrifugation of fresh latex at $39000 \times g$ for 20 min but some RNA (15–20% of the total RNA) is found regularly in the bottom (lutoïd) fraction.

High activities of polyphenol oxidase and acid phosphatase are recovered in the bottom fraction. This fraction is thus heterogenous. It contains not only lutoïds but also Frey-Wyssling particles and likewise some contamination origi-

Table 1. Distribution of RNA content and acid-phosphatase and polyphenol-oxidase activities between lutoïd and serum fractions from *Hevea brasiliensis* latex

RNA content and enzymatic activities are expressed per ml of collected latex (in μg for RNA, in μmol of *p*-nitrophenol per min for acid phosphatase, and in arbitrary units per min for polyphenol oxidase). Content or activity of each fraction is also expressed in percent of the total content (or activity) recovered. Results from 6 experiments, with standard deviation calculated for a probability of 99%.

| | Latex | Rubber fraction | | Serum fraction | | Lutoïd fraction | |
|---|---|---|---|---|---|---|---|
| | Activity or content | Activity or content | % of total latex activity or content | Activity or content | % of the total latex activity or content | Activity or content | % of total latex activity or content |
| RNA | 18.9 ±1.5 | 2.1 ±0.3 | 11.2 | 13.4 ±0.6 | 70.6 | 3.4 ±0.6 | 18.2 |
| Acid phosphatase | 0.67±0.11 | 0.02±0.01 | 2.8 | 0.13±0.04 | 19.5 | 0.52±0.05 | 77.7 |
| Polyphenol oxidase | 1.13±0.21 | 0.05±0.03 | 4.2 | 0.32±0.07 | 28.1 | 0.76±0.10 | 67.7 |

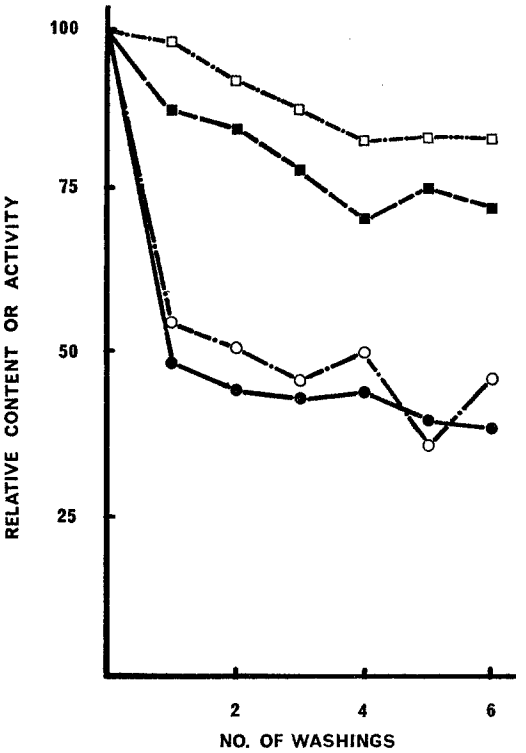


Fig. 1. Effect of the washing procedure on the removal of contamination from the lutoïd fraction. Relative RNA content and acid-phosphatase activity are expressed in percent of the initial content or activity in the crude lutoïd fraction. Solid symbols = washing without EDTA, open symbols = washing with EDTA; circles ( ●, ○) = RNA content, squares (■, □) = acid-phosphatase activity. Results represent the average of 8 experiments

nating from serum, *e.g.* ribosomes bound to the lutoïd membrane. It is therefore necessary to specify the association of the RNA found in the lutoïd fraction with the structures present in this fraction.

### *Effect of Washing Procedure on the Removal of Contamination from the Lutoïd Fraction*

When the crude lutoïd pellet is washed with Tris-mannitol buffer (Fig. 1) more than 40% of the lutoïd RNA are removed during the first washing whilst 10% of the acid phosphatase is solubilized. RNA and acid-phosphatase activity losses decrease with repeated washings. Thus, after 3 washings, the relative RNA content and acid-phosphatase activity of the lutoïd fraction remained constant: about 40–50% for RNA and 75–85% for the hydrolase activity.

Addition of EDTA to the washing medium does not increase the amount of RNA removed from the lutoïd fraction although this chelating agent can released RNA from membranes (see Sabatini *et al.*, 1972). Repeated washings, although they eliminate most of the subcellular particles which were broken during the

preparation of the lutoïd fraction, also do not alter the amount of RNA recovered from this fraction. Free RNA originating from some contaminants of the lutoïd fraction, *e.g.* rough endoplasmic reticulum or other membraneous components of the serum, is eliminated during the two first washings. Under these conditions, it is difficult to assume that residual lutoïd RNA is adsorbed to the lutoïd membrane or another structure present in the fraction.

### *Insensitivity of Lutoïd RNA to Ribonuclease*

When the lutoïd fraction is incubated in the presence of pancreatic RNase at 37°, some degraded RNA appears in the supernatant after 30 min. The conditions of incubation do not appreciably modify the latency. Thus, some RNA present in this fraction can be digested by exogeneous RNase. However, this release of alcohol-soluble material, which proceeds at a declining rate for 30 min, amounts to only 3.7% of the total RNA in the fraction (Fig. 2).

On the other hand, the lutoïd RNA is degraded by pancreatic RNase to a greater extent when the lutoïd preparations are destabilized, the mode of destabilization being important in determining the extent of digestion. The lutoïd fraction can be labilized by mechanical treatment or by osmotic treatment. After 1 h incubation, 63.1% of RNA is digested after mechanical destabilization and 43.4% after osmotic disruption. No further digestion is found when the incubation time is extended.

The solubilization of acid-phosphatase activity is also greater after destabilization of the lutoïd fraction. The latency for this activity is 78.4% for the mechanical treatment and 59.0% for the osmotic one.

Thus, a relation exists between the state of some particles in the lutoïd fraction and the extent of the hydrolysis of this RNA by an exogeneous RNase. Unless these particles are destabilized, RNA is not degraded. It seems that this RNA is not accessible to RNase. We suppose that it is present in a particle which protects it from digestion.

The relative inefficiency of the hydrolase action in obtaining a complete digestion of the RNA present is the result of the procedure used. RNase is not added during the preparation of destabilized lutoïds but after. It is thought that the disrupted membranes form some single-membrane vesicles rapidly, *de novo*. Such a formation of vesicles from disrupted membranes has been often noticed and is not specific to the lutoïds. But if it takes place the RNA found in the fraction in question is not exposed to added RNase and cannot be degraded as far as it is constitutive of the membraneous structure.

### *Nature of the Particle that Contains RNA*

The lutoïd fraction is heterogeneous, containing predominantly lutoïds and Frey-Wyssling particles, but also some contaminants originating from serum and proliferating bacteria originating from the *Hevea* bark and collected during the tapping procedure.

The contribution of bacteria to the RNA of the lutoïd fraction was studied in four different sources of latex in our experimental conditions of collection (Table 2). As a rule, not more than 1% of the latex RNA may be originating from bacterial contamination.

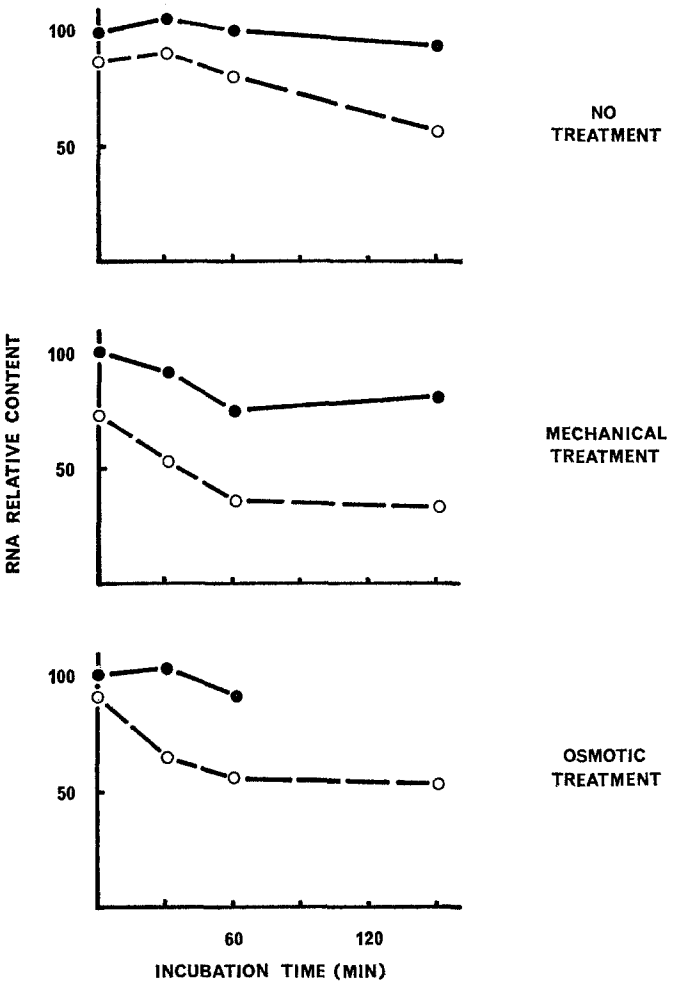


Fig. 2. Effect of pancreatic RNase on the RNA content of the lutoïd fraction. Results are expressed as percent of the initial content of the non-treated lutoïd fraction. Solid lines = untreated lutoïds; broken lines = RNase-treated lutoïds. Results represent the average of 10 experiments

Treatment with digitonin did not liberate RNA from the lutoïd pellet (bottom fraction) (Table 3). However, this detergent solubilizes a substantial part of polyphenol-oxidase activity (44.1%). This is in agreement with the fact that it is known to destabilize specifically the membrane of the Frey-Wissling particles (Coupé *et al.*, 1972). In contrast, acid-phosphatase activity is not affected. Thus, it appears that it is necessary to solubilize the acid-phosphatase activity in order to liberate RNA present in the lutoïd fraction. In other words, a relation exists between the integrity of the lutoïds (for which acid phosphatase is a marker) and the liberation of RNA in the supernatant (Fig. 3). Thus, it is probable that the RNA found in the lutoïd fraction is localized in the lutoïd organelle.

Table 2. Contribution of bacterial contamination to RNA content of lutoid fraction
The bacterial proliferation on the tapping pannel varying with the physiological conditions of tapped tree, four sources of latex are tested. Results, expressed per ml of latex, represent the average of three experiments with standard deviation calculated for a probability of 95%.

| Source of latex | Lutoïd RNA content of latex ($\times 10^{-6}$ g) | No. bacterial colonies ($\times 10^{6}$) | Bacterial RNA content of latex ($\times 10^{-6}$ g) | Estimate of bacterial RNA as % of total lutoïd RNA |
|---|---|---|---|---|
| 1 | $2.11 \pm 0.08$ | $0.29 \pm 0.02$ | 0.146 | 6.93 |
| 2 | $9.34 \pm 0.10$ | $0.51 \pm 0.08$ | 0.254 | 2.70 |
| 3 | $6.24 \pm 0.10$ | $0.08 \pm 0.01$ | 0.004 | 0.06 |
| 4 | $4.26 \pm 0.12$ | $0.09 \pm 0.01$ | 0.004 | 0.09 |

Table 3. Effect of different destabilizing factors on RNA content of the lutoïd fraction
The destabilized lutoïd suspension is centrifuged at $39000 \times g$ for 15 min. Content or activity recovered in the sediment is expressed as percent of the initial content or activity present in the lutoïd fraction. Results represent the average of 8 experiments.

| Treatment applied to lutoïd fraction | Content or activity recovered in the $39000 \times g$ sediment (%) | | |
|---|---|---|---|
| | RNA | Acid phosphatase | Polyphenol oxidase |
| None | 87.6 | 95.4 | 85.0 |
| Mechanic shock | 37.1 | 14.4 | — |
| Osmotic shock | 45.2 | 40.2 | — |
| Triton X-100 (final concn. 1%) | 26.7 | 4.9 | — |
| Digitonin (final concn. 0.5%) | 81.0 | 91.6 | 55.9 |

This conclusion is supported by an analysis of heterogeneity of this fraction by linear sucrose density-gradient centrifugation (Marin *et al.*, 1974). This procedure separates acid-phosphatase activity from polyphenol-oxidase activity and thus lutoïds from Frey-Wissling particles. RNA distribution is identical with the acid-phosphatase profile but very different from the polyphenol-oxidase one. The association between RNA content and acid-phosphatase activity is also evident.

The density of lutoïds and thus their sedimentation through a sucrose gradient (Fig. 4) can be modified by Neutral red. We notice also the polydispersity characteristics of the *Hevea* lysosomal compartment. Nevertheless, the RNA distribution remains approximatively parallel to the acid-phosphatase activity profile. This result confirms that RNA present in the lutoïd fraction is mainly associated with the lutoïd organelle and not with some contaminants present in this fraction.

## Discussion and Conclusion

The experiments described in this paper show that the RNA which has been found in *Hevea* latex is present mostly (about 70%) in the serum fraction but a

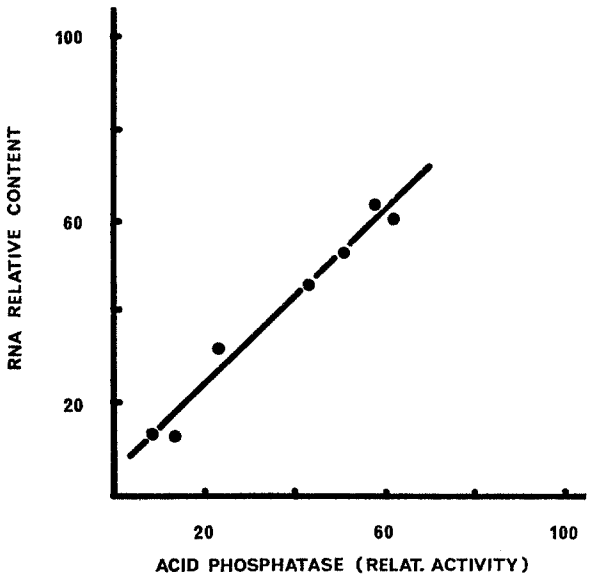


Fig. 3. Relation between RNA and acid phosphatase activity present in the sediment obtained from different destabilized lutoïd fractions by differential centrifugation. The relative RNA content and acid phosphatase activity of the $39000 \times g$ sediment are expressed as percent of the initial RNA content and acid-phosphatase activity of the untreated lutoïd fraction. Results represent the average of 8 experiments

smaller fraction (*ca.* 18%) is consistently associated with the lutoïd fraction. Our investigations provide some information about the latter RNA.

The rationale of our approach is based on the recognition that lutoïds are the principal cellular structures present in the lutoïd fraction, and that a relation appears to exist between RNA content and acid-phosphatase activity of the lutoïds.

The lutoïd RNA is largely insensitive to RNase action. It is necessary to destabilize the lutoïd fraction to solubilize its RNA (as well as acid phosphatase) and to permit its degradation by an exogeneous RNase. Nevertheless, the digestion remains incomplete because revesiculation cannot be avoided during the destabilization procedure and limits the RNase digestion of the RNA. This result indicates that this RNA is protected from RNase hydrolysis while present in an intact particle of the lutoïd fraction. Since RNA parallels strictly acid phosphatase activity, it is probable that the structure which contains the RNA is the lutoïd organelle. Isopycnic centrifugation of the lutoïd fraction confirms the presence of RNA in this organelle.

Not all the RNA found in the lutoïd fraction is present in the lutoïd organelle. Some contribution is made by bacterial contamination which occurs during the tapping procedure and by the presence of some serum in the lutoïd fraction. The bacterial contamination is however not great enough to account for more than 7% of the total RNA present in the lutoïd fraction (Table 3). The RNA which comes from the serum contamination is removed by repeated washings.

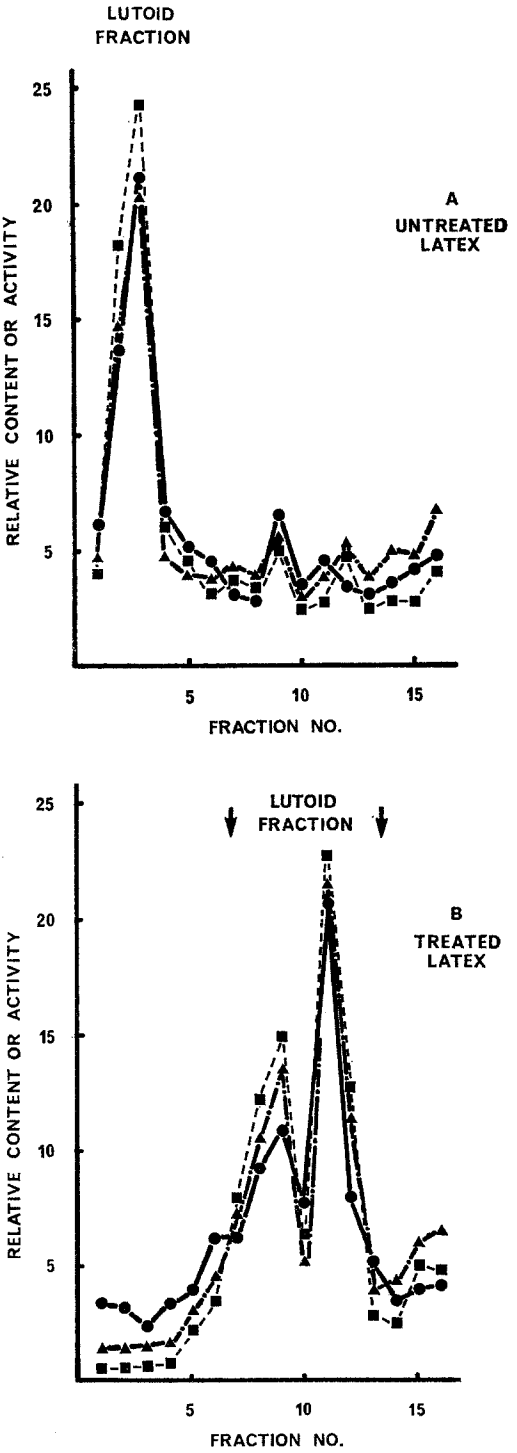


Fig. 4A and B. Influence of Neutral red on the sedimentation of lutoïds through sucrose gradient. The relative content or activity is expressed in percent of the total recovered content or activity. ■ Acid-phosphatase activity; ▲ proteins; ● RNA. (A) non-treated lutoïds, (B) lutoïds charged with Neutral red

It is not surprising to find in the lutoïd organelle some RNA. The lutoïd is a single-membrane structure having some vacuolar characteristics (Ribaillier *et al.*, 1971) and some lysosomal properties (Pujarniscle, 1969). At the present time, the occurence of RNA has been established for many such particles, namely glyoxysomes (Gerhardt and Beevers, 1969; Ching, 1970), vacuoles (Matile and Wiemken, 1967) and lysosomes (Matile, 1968; Pitt and Galphin, 1973). However, RNA extraction from these organelles is difficult because the preparation of large quantities of a purified fraction is not very workable in most materials. A detailed study is possible with lutoïd RNA. The lutoïd fraction is easy to prepare in abundant quantities from *Hevea brasiliensis* latex and the amount of lysosomal RNA extracted from these organelles can be sufficient to elucidate its nature and function.

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