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Significance of Non-staining White Zones in Starch Gel Electrophoresis

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Starch gel electrophoresis of B- and C-sera has occasionally shown non-staining white zones in addition to blue protein bands staining with Naphthalene Black (Amido Schwarz) staining reagent. These white zones have been identified as ribonucleic acid by their ultraviolet absorption characteristics, action of pancreatic ribonuclease and staining properties with acridine orange and toluidine blue. In the authors' view this negatively staining property of DNA and RNA with Naphthalene Black on starch gel is a new phenomenon which has not hitherto been reported in literature.

In starch gel electrophoresis of proteins, visualisation of the protein bands is commonly made with Naphthalene Black (Amido Schwarz) staining reagent whereby the protein bands appear dark blue on a pale blue background. The pale blue background staining is due to proteins which are originally present in the starch and it can be prevented by treatment of the starch prior to electrophoresis with a bacterial protease (ROBINSON AND PIERCE, 1963).

Hevea latex is a colloidal suspension of rubber and other particles in an aqueous medium. Ultracentrifugation separates freshly tapped latex into three well defined zones (COOK AND SEKHAR, 1953), namely, a light rubber phase, an aqueous phase — C-serum — and a heavy 'bottom fraction' composed largely of membrane-bounded particles called lutoids (HOMANS AND VAN GILS, 1948); from the 'bottom fraction' a serum — 'B-serum' — may be prepared by disruption of the membrane (HSIA, 1958). The two sera, B and C, show quite characteristic protein patterns when subjected to starch gel electrophoresis.

In the course of studies on B- and C-sera, the authors have observed non-staining white zones in addition to dark blue protein bands on starch gel electropherograms. In these white zones the usual pale blue background staining was absent. Sometimes these zones were irregular and broad but their migration was fairly constant and reproducible. However, in many samples of B- and C-sera, they were not observed, possibly due to variation in latex composition from day to day.

This paper describes the investigations to ascertain the significance of these white zones and shows that they are due to nucleic acids.

EXPERIMENTAL

Latex was collected from a group of trees of clone RRIM 600 (tapped on S/2.d/2.100% system) in Field 48 of the R.R.I.M. Experiment Station. Glass vessels surrounded by melting ice were used for collection of the latex. Centrifugation was carried out in a Spinco Model L ultracentrifuge using No. 21 rotor which was not pre-chilled. Time and speed of centrifugation were as described by MOIR AND TATA (1960). After centrifugation, C-serum was removed from the tubes and the 'bottom fractions' were pooled. B-serum was prepared from the pooled 'bottom fraction' by methods similar to those described by KARUNAKARAN *et al.* (1961).

Starch gel electrophoresis was performed using 'Starch Hydrolysed' manufactured by the Connaught Medical Research Laboratories, Toronto, Canada. Gels were prepared at 1¼ times the concentration recommended by the manufacturers for the particular batch of starch being used. The overall procedure

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was as described earlier (TATA AND MOIR, 1964) with minor modifications.

Preparations of RNA from B- and C-sera were made by the method described by LITWACK (1960) for isolation of RNA from liver, which is a modification of the method of KIRBY (1956). Estimations of RNA were made by the orcinol method of DEAN AND HINSHELWOOD (1960). A standard RNA curve was constructed with commercial RNA (yeast), purchased from Koch Light & Co., England. Protein estimations were made by a modification of the method of LOWRY *et al.* (1951) using the Folin Ciocalteu reagent.

RESULTS AND DISCUSSION

Figure 1 illustrates starch gel electropherograms of B- and C-sera showing the white zones. The white zones were always anionic at the pH of electrophoresis, *i.e.*, pH 8.2 and so far the authors have not observed white zones on the cathode side of the origin on starch gel. The white zone near the origin was observed in B-serum, and the fast moving zone near the anode end of the gel was observed in C-serum.

A close examination of the white zones revealed that they were areas which had not taken up the Naphthalene Black reagent. The background staining in the white zones was always absent but sometimes it was possible to detect stained protein bands within the white zones.

In view of the observation of ROBINSON AND PIERCE (1963), these white zones might be considered as proteolytic enzymes in B- and C-sera which were capable of attacking the residual starch protein but not the proteins of either B- or C-serum which gave the dark blue bands. However, evidence presented below seems to show that these white zones were due to nucleic acids.

When commercial samples of DNA (calf thymus) and RNA (yeast) (purchased from Koch Light & Co., England) were subjected to starch gel electrophoresis, very marked white zones appeared on staining the gel with Naphthalene Black reagent *(Figure 2)*.

Both white zones were anionic and the one due to RNA had migrated some distance from the origin whereas the one due to DNA had remained close to the origin. This negatively staining property of DNA and RNA with Naphthalene Black reagent on starch gel has not been hitherto reported in the literature as far as the authors are aware.

Naphthalene Black reagent is rendered colourless by sodium borohydride (a reducing agent) but is unaffected by hydrogen peroxide (an oxidising reagent). It seems unlikely that

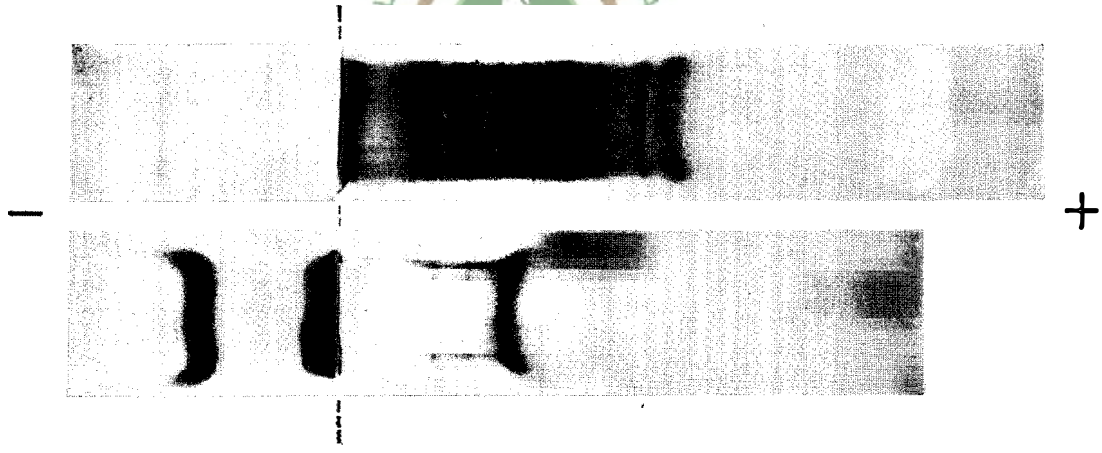

Figure 1. Starch gel electropherograms of C-serum (top) and B-serum (bottom) showing white zones in addition to the protein bands staining with Naphthalene Black reagent. Electrophoresis was performed for 6 hours.

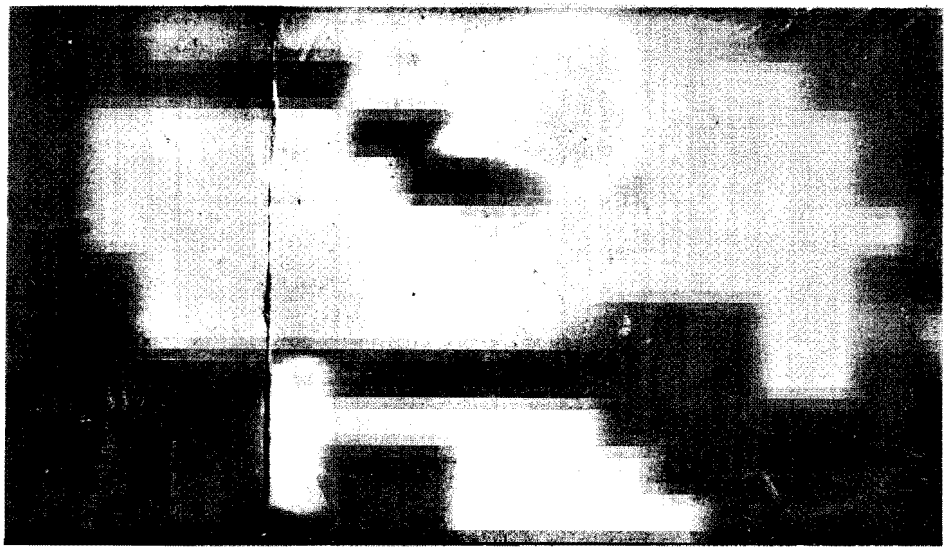

Figure 2. White zones obtained by electrophoresis: top — RNA (yeast) and bottom — DNA calf (thymus). Electrophoresis was performed for 3 hours.

either DNA or RNA has a reducing action on Naphthalene Black. Experiments employing various conditions of pH, concentration and temperature showed that neither DNA nor RNA decolourised even a very dilute solution of Naphthalene Black.

A batch of 'Starch Hydrolysed' was found to contain approximately 0.6% residual protein. This protein must have been denatured during the acid hydrolysis process (SMITHIES, 1955). It seems likely that both DNA and RNA combine with the residual protein in starch and render the dye binding sites of the latter unavailable to Naphthalene Black. Once this combination had taken place, the area occupied by the nucleic acid would appear white as compared with the rest of the gel which would have the lightly stained background due to the uncombined residual protein. Experimental evidence to show that DNA and RNA had some action on the residual protein of starch was obtained when a sample of 'Starch Hydrolysed' was mixed with the commercial samples of DNA and RNA (5 mg of nucleic acid to 1 g of starch) and gels were prepared from the mixtures. A control gel was also prepared from the same sample of starch. When these three were stained with Naphthalene Black reagent, gels containing the nucleic acids were found to be colourless as compared with the pale blue control gel.

RNA Preparation from B- and C-Sera

The presence of RNA in *Hevea brasiliensis* latex has been shown by MCMULLEN (1959 and 1962). The authors have isolated RNA from B- and C-sera using 90% aqueous phenol as described by LITWACK (1960). On analysis, a preparation from B-serum was found to be approximately 10% RNA; that from C-serum was found to be approximately 35% RNA, as determined by the orcinol method (DEAN AND HINSHELWOOD, 1960).

Starch Gel Electrophoresis of RNA Preparations

RNA preparations from B-serum showed four white zones and those from C-serum showed three. In order to ascertain the identity of these white zones, the gel was sliced transversely after electrophoresis into two slices of equal thickness. One slice was stained with Naphthalene Black reagent to locate the white

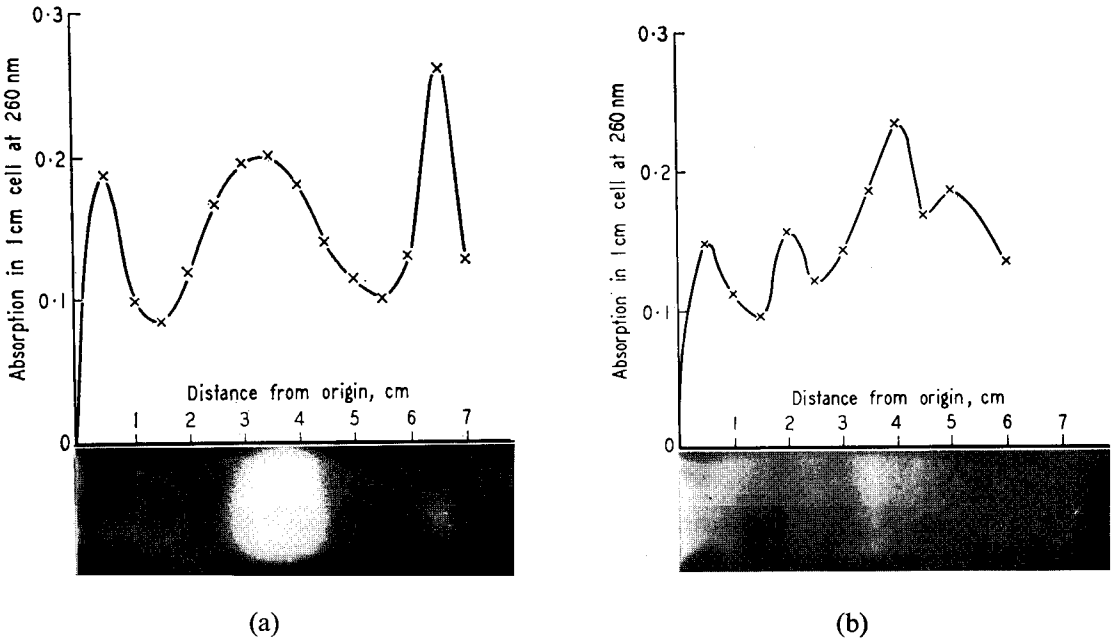

Figure 3. Elution curves and photographs of stained gels of RNA preparations from (a) C-serum and (b) B-serum. Electrophoresis was performed for 3 hours.

zones, whereas the other slice was cut into 0.5 cm sections parallel to the sample slot. The sections were extracted with 0.5 *N* perchloric acid and the absorption of each extract at 260 nm was measured. *Figure 3* consists of photographs of the stained gels showing the white zones and the corresponding absorption curves at 260 nm.

Effect of Ribonuclease

The effect of treating commercial RNA (yeast) with pancreatic ribonuclease (Sigma Chemicals, U.S.A.) is illustrated in *Figure 4(a)*. The RNA shows a white zone on starch gel but after treatment with ribonuclease it is decomposed and consequently does not give a white zone. [For this experiment a few mg of RNA (yeast) were incubated with a trace of ribonuclease before subjecting the mixture to starch gel electrophoresis.]

Ribonuclease produced a similar effect on the RNA preparations from B- and C-sera. Each preparation was apparently decomposed in the expected manner on treatment with ribonuclease since the main white zones disappeared after the treatment. This effect is illustrated in *(b)* and *(c)* of *Figure 4*.

In *(b)* and *(c)* of *Figure 4*, the faint white zones which persist after treatment of the RNA preparation with ribonuclease are due to contamination of the preparation with phenol, which by itself gives white zones on starch gel.

The authors have not been able to obtain evidence of the presence of DNA in either B- or C-serum and their attempts to estimate DNA in either B- or C-serum by a modified Dische method of DEAN AND HINSHELWOOD (1960) gave negative results. Also, their RNA preparations from B- and C-sera were not affected on treatment with deoxyribonuclease (Koch Light & Co., England), *i.e.*, the behaviour of both the preparations on starch gel was unchanged after treatment with deoxyribonuclease.

Ultraviolet Absorption Characteristics

Figure 5 illustrates the ultraviolet absorption

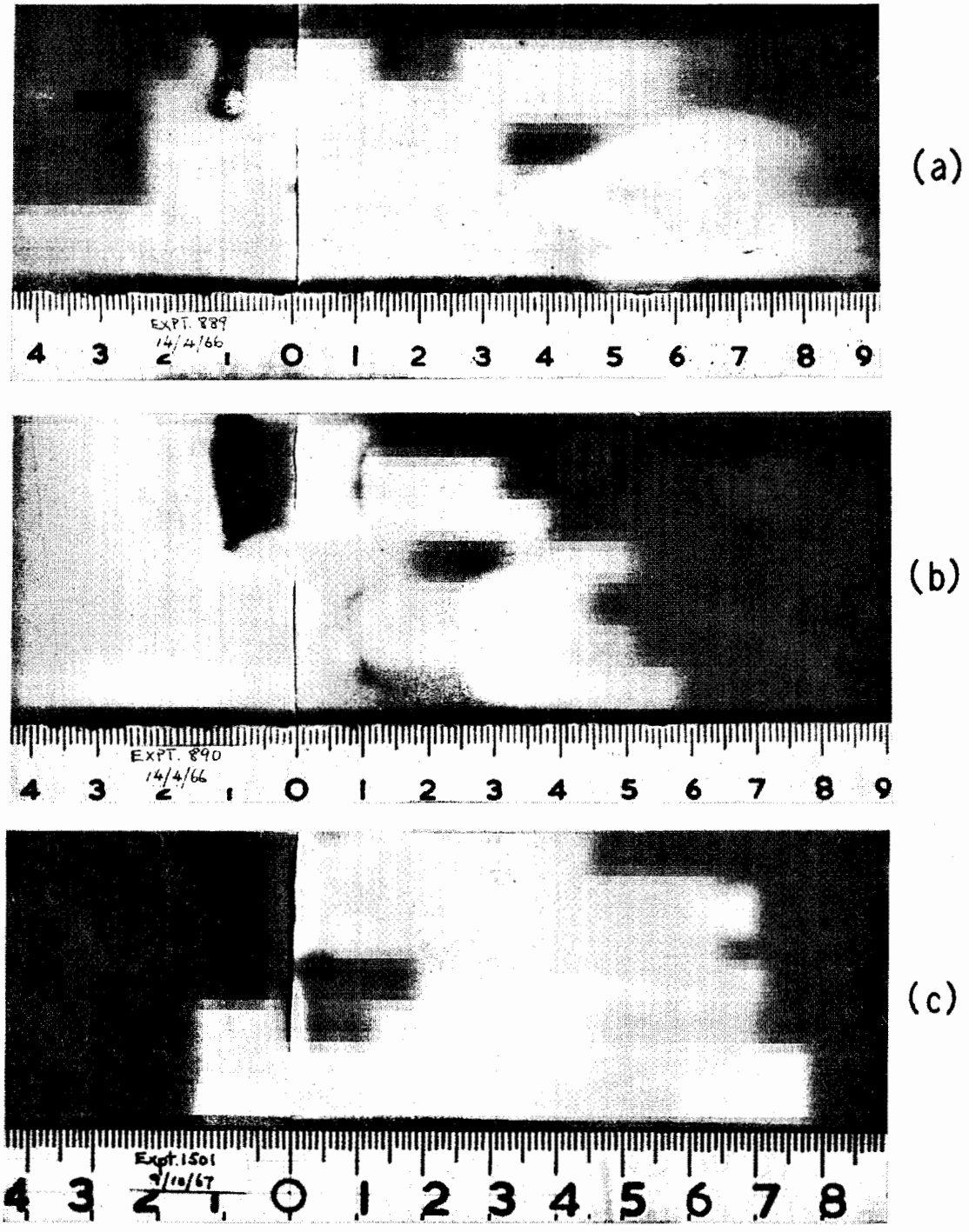

Figure 4. Effect of pancreatic ribonuclease on (a) commercial RNA (yeast), (b) RNA preparation from B-serum and (c) RNA preparation from C-serum. In each photograph, the mixture of ribonuclease and the nucleic acid is shown at the top and the untreated nucleic acid at the bottom. The dark stained band on the left of the origin is due to ribonuclease. Electrophoresis was performed for 3 hours.

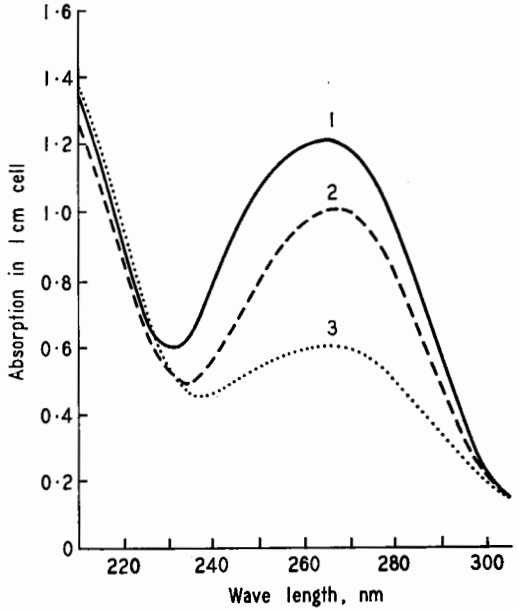

Figure 5. Ultraviolet absorption spectra of 1 *— RNA (yeast),* 2 *— RNA preparation from C-serum, and* 3 *— RNA preparation from B-serum, showing minima at 230 – 235 nm and maxima at 260 – 265 nm.*

characteristics of a commercial sample of RNA (yeast) and of RNA preparations from B- and C-sera. The minimum and maximum absorptions at 230–235 nm and 260–265 nm respectively are the characteristic absorptions of nucleic acids.

Staining with Acridine Orange and Toluidine Blue

Acridine orange and toluidine blue have both been used to stain RNA on gels after electrophoresis (RICHARDS *et al.*, 1965; BENEY AND SZÉKELY, 1966). When the authors' preparations were electrophoresed on starch gel, the white bands due to RNA on one slice of the gel (stained with Naphthalene Black) were found to correspond with bands on the duplicate slice stained with acridine orange. An exactly similar result was obtained with toluidine blue. Toluidine blue staining was visible in ordinary light; bands stained with acridine orange were seen as green-fluorescent under ultraviolet lamp ('Chromatolite', from Messrs. Hanovia Lamps, Slough, Bucks, U.K.)

CONCLUSION

From the experimental evidence presented here, it is concluded that the white bands in B- and C-sera are due to ribonucleic acid and are not proteolytic enzymes. Perhaps the most convincing evidence was provided by the action of pancreatic ribonuclease on the white bands. The ultraviolet absorption characteristics and staining with acridine orange and toluidine blue provide additional evidence to support the above conclusion.

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DISCUSSION

Discussion on this paper is included in MOIR (1969), see page 416.