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A Rapid Assay for the Incorporation of Isopentenyl Diphosphate in Rubber Biosynthesis

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A rapid, sensitive assay for comparing relative rates of rubber biosynthesis in vitro *is presented. This method is considerably faster than those previously described. Due to its simplicity, the assay is appropriate for monitoring the purification of factors involved in rubber biosynthesis. However it is necessary to be wary of the possible effects of certain ions such as Mg^{2+}, Na^{+} and phosphates, which at certain concentrations considerably affect the rate of rubber biosynthesis.*

Rubber formation in plants results from the *cis*-1,4-polymerisation of the isopentenyl units of isopentenyl diphosphate (IDP), catalysed by the enzyme rubber transferase (E.C.2.5.1.20). Polymerisation occurs head-to-tail and requires a prenylallylic pyrophosphate as initiator[1–4], *in-vitro*, both *cis* and *trans* compounds can serve this purpose[2]. This enzyme reaction is the characteristic step in the synthesis of rubber, which occurs in over two thousand plant species. The transferase is tightly bound to the rubber particles[1–5] ; views that there is also a soluble form of the enzyme[6–8] have recently been shown to be most unlikely[9]. On the other hand, enzymes which catalyse the synthesis of the required initiator molecules, do occur in soluble form[9].

Assays for rubber transferase have been used since the 1960s, and are based on the incorporation of radiolabelled precursors into rubber. Several assays for the enzyme have been published[2–4,6–8,10], but all involve the same basic procedure. Purified rubber particles, source of the rubber transferase, plus any fractions under investigation were incubated in an appropriate buffer with [^{14}C]-IDP, a thiol, a divalent metal ion, and sometimes an initiator molecule. The reaction was stopped by the addition of EDTA, and 'carrier' rubber sometimes added to help the coagulation of rubber prior to purification. Earlier methods for measuring amount of [^{14}C]-IDP incorporation[6,7,10], involved time-consuming and tedious processing of the reaction mixture to remove the unreacted labelled IDP and its metabolites. Light and Dennis[8] considerably improved the procedure by using filtration to trap rubber particles, following [^{14}C]-IDP incorporation. However, even this approach required each filtered sample to be processed separately, and was thus very time-consuming

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for multiple samples. For the work on purification and characterisation of protein(s)/ enzyme(s) involved in rubber biosynthesis, or where there is a need to assay large numbers of samples from chromatography runs as quickly as possible before proceeding to the next purification step, the existing assays were found unwieldy and time-consuming. The basic assay described by Cornish and Backhaus[4] was therefore, carefully examined, and where necessary, each step modified to reduce the time taken. The use of a Bio-Dot (Bio-Rad) filtration apparatus was introduced, to allow the simultaneous filtering and processing of up to 96 samples at once. Because of these changes, the assay which is presented here, is capable of comparing the relative synthetic rates of 96 samples in under five hours.

MATERIALS AND METHODS

Preparation of C-serum Solids

Latex was collected from *Hevea brasiliensis* RRIM 600 trees tapped ½S d/2 in a flask chilled in melting ice. It was then centrifuged in a Sorvall RC-5C in the SM-34 rotor for two h at 43 000 g (maximum) at 4°C. The latex separated into four main phases; the top-most is the light rubber phase followed by a thin yellowish layer which contains the Frey-Wyssling complexes. The clear C-serum equilibrates in the middle of the tubes, and the heavy and yellowish lutoid fraction at the base[11]. C-serum from all tubes was pooled and centrifuged again at the same speed for an hour to remove further rubber. The resulting, much clearer C-serum, which still contained small rubber particles, was then freeze-dried to give the yellowish C-serum solids (*ca.* 7 g per 100 ml). This material invariably contains small amounts of rubber particles and is highly active in synthesising rubber from IDP[2]. The activity is retained for many years on storage at -20°C.

Preparation of Washed Rubber Particles

A reconstituted solution of C-serum solids (6 ml of 25%) in 250 mM Tris-HCl buffer pH 8.0, containing 5 mM 2-mercaptoethanol was loaded onto a Sephadex G-150 column (80 cm × 2.6 cm), equilibrated with the same buffer, which was also used for elution (0.5 ml/ min). 5 ml fractions were collected and their absorbance determined at 280 nm. Three main peaks were observed, Fractions A, B and C (*Figure 1*), as found previously[2].

The cloudy Fraction A contains mainly rubber particles and proteins with molecular weights of more than 300 kDa (the upper cut-off of the column). The constituent fractions were pooled and concentrated up to three times to about 10 ml by ultrafiltration using a membrane filter (Amicon) with a cut-off of 3000 Da, to give the washed rubber particles (WRP) used in the assay. 50 µl of the concentrate WRP were used per 200 µl of incubation mixture. This is approximately equivalent to 7.5 mg of C-serum solids, or 30 µl of the 25% solution.

'Carrier' Rubber

'Carrier' rubber was prepared from high ammonia *Hevea brasiliensis* latex concentrate of 60% rubber content. For use as 'carrier' rubber, this latex was diluted to 1% rubber with distilled water.

Precursor Compounds

Isopentenyl diphosphate (IDP), neryl diphosphate (NDP) and *trans,trans*-farnesyl diphosphate (FDP) were synthesised by the method of Davisson *et al.*[12]. Analysis by

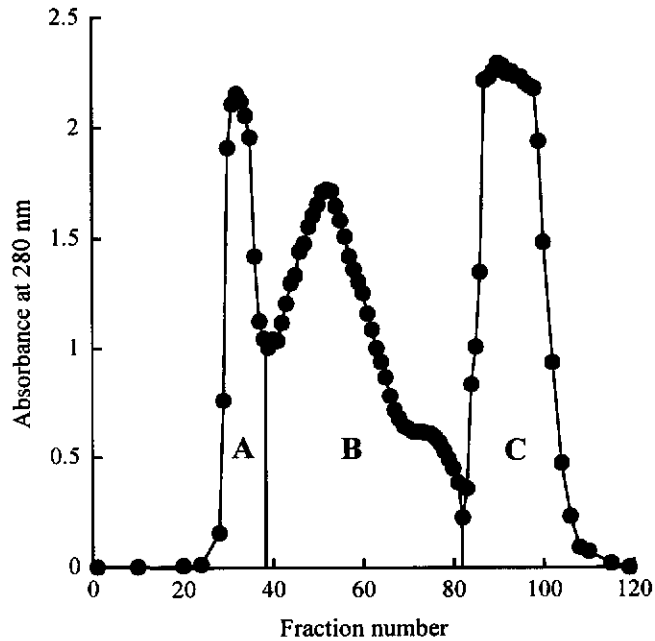


Figure 1. Gel-filtration chromatography of 6 ml 25% reconstituted C-serum solids on Sephadex G-150 (80 × 2.6 cm), equilibrated with 250 mM Tris-HCl, pH 8.0 containing 5 mM 2-mercaptoethanol; elution was with the same buffer and 5 ml fractions were collected; flow-rate 0.5 ml per minute. Fraction A: mainly rubber particles; Fraction B: mainly proteins; Fraction C: compounds of molecular weight of less than 5000 Da.

^{1}H-NMR and thin layer chromatography (TLC) showed that the materials were not pure, though the desired compound was the main component; the *trans-trans*-farnesyl diphosphate contained geometric isomers as well as other impurities.

In calculating concentrations it has been assumed that the compounds were unhydrated triammonium salts and the purity, 100%. [1-^{14}C]-IDP, specific activity 99 140 dpm per nmol, was obtained from Amersham, UK.

Purification of Labelled Rubber by Gel-filtration

To check that the membrane-filtration method described below, yields radiochemically pure rubber, samples of incubation mixtures containing labelled rubber, were chromatographed on a Sephadex G-150 column (50 cm × 1.6 cm), equilibrated with 250 mM Tris-HCl buffer, pH 8.0. Each incubation (200 µl) contained 50 µl WRP, 0.7 mM NDP, and other additions as described below in Final Assay Conditions. After incubation (0, 60 and 180 min), 5 × 200 µl samples were chromatographed using the same buffer for elution. 1.5 ml fractions were collected until the absorbance at 280 nm returned to zero, and the radioactivity of each determined; rubber particles elute in the void volume. This procedure is known to separate the rubber particles, on which incorporation takes place[10] from residual IDP and its low molecular weight metabolites[13].

To Calculate the Absolute Amount of IDP Converted to Rubber

The absolute amount of IDP converted to rubber in the assay was calculated[14] as follows:

Amount of ^{14}C-IDP added to the incubation mixture is given in dpm, *e.g.* *X* dpm;

Amount of 'cold', IDP added to the incubation mixture is given in µmole, *e.g.* *Y* µmole.

Therefore, the Specific Activity of IDP used in the incubation assay is:

$$\frac{X \text{ dpm}}{Y \text{ µmole}}$$

If the incorporation of ^{14}C-IDP at *Y* µmole IDP is observed to be *Z* dpm, therefore, the amount of IPP converted to rubber is:

$$\frac{Z \text{ dpm}}{\text{Specific Activity of IDP used}}$$

or

$$\frac{Z \text{ dpm}}{\dfrac{X \text{ dpm}}{Y \text{ µmole}}}$$

or

ZYX^{-1} µmole of IDP.

Basic Assay

Unfractionated C-serum solids were used in the basic assay[4]. This material, reconstituted to a concentration of 25% (w/v) with 250 mM Tris-HCl buffer pH 8.0, was centrifuged as above to remove large, or coagulated rubber particles. The assay mixture contained 40 µl of the centrifuged C-serum solution, 48.0 nmol (4 791 650 dpm)[1-^{14}C]-IDP, 1 mM $MgSO_4$, 50 mM DTT and 50 mM Tris-HCl, pH 7.5; total volume 5 ml. After incubation in a shaking water-bath at 25°C, 400 µl of the mixture was removed and mixed with 50 µl of 0.3 M EDTA, pH 8.0, to stop the reaction. 'Carrier' rubber (20 µl) were added, and the mixture filtered through a 2.5 cm diameter nitrocellulose (0.2 µm) filter membrane (Millipore) on a filtration assembly (Millipore). After heating at 37°C overnight to coagulate the rubber, the filters were placed in scintillation vials, washed with 3 ml of 1 M HCl for 15 min, followed by three washes with 3 ml of 95% ethanol for 15 min, and then dried under an infrared lamp.

Scintillation fluid (Liquiscint by National Diagnostic Inc., U.S.A.) was then added and the radioactivity measured on the liquid scintillation counter (LKB Wallac 1219 Rackbeta). *Figure 2* illustrates the conversion of IDP to rubber under the above conditions, over a period of four h. As found by Cornish and Backhaus[4], some labelled carbon was present on the filters even when zero-time incubations were processed. This is most likely [^{14}C]-IDP, not removed by the purification procedure. The amount was small, usually around 0.5% of the IDP incorporated into rubber. This 'blank' value was determined in all experiments, and used to correct the data. No label was retained by the filters in the absence of rubber particles. This basic assay took more than 24 h to complete and only about ten samples could be adequately handled at one time.

RESULTS AND DISCUSSION

Modification of the Basic Assay

Use of 'carrier' rubber. When a mixture incubated for three hours was put through the purification method without 'carrier' rubber, 500 dpm/ml of reaction mixture were trapped on the filter, whereas in the presence of 50 µl of 'carrier' rubber, 2200 dpm/ml were retained. 'Carrier' rubber, therefore, considerably enhances the amount of synthesised rubber trapped. Increasing the amount of rubber from 50 µl to 100 µl/ml of incubation mixture, resulted in a slight (5%) increase in trapped radioactivity, but this also increased the 'blank' counts (from 455 dpm to 605 dpm/ml), and also slowed the filtration rate. A volume of 50 µl 'carrier' rubber/ml of incubation mixture was therefore chosen for use in subsequent experiments.

Choice of filter and heating time. Using nitrocellulose filters (0.2 µm), approximately 20% more counts were retained than when glass-fibre ones (0.7 µm) were used. This was not surprising, given the respective pore sizes. However, much faster flow-rates were achieved with glass-fibre, and this was therefore preferred.

In the method of Cornish and Backhaus[4], the membrane filter is heated at 37°C to help coagulate the rubber. In order to reduce heating time, the effect of heating at 110°C for 10 min and 60 min, was investigated. Both treatments gave higher recoveries of radioactive rubber than 37°C treatment (*Figure 3*). Since 60 min heating only gave a 10% increase in recovery over that of 10 min, in the interest of time-saving, 10 min heating at 110°C was subsequently employed.

Effect of the concentration of C-serum. As might be expected, increasing the final concentration of the reconstituted C-serum in the incubation mixture, from 1% to 2%, resulted in significantly enhanced incorporation (*Figure 4*), but at a concentration of 4%, there was little further effect. Increasing the C-serum concentration also resulted in lower filtration rates. The C-serum was therefore used at 2%.

Use of microtitre plates. Use of 96-well microtitre plates was introduced to overcome the problem of having too many samples to be assayed at the same time. Due to the limited size of the wells, the final volume of the incubation mixture was scaled down to 200 µl. A multichannel pipette was used to assist the mixing and transferring of incubation mixtures.

Addition of unlabelled IDP. In order to eliminate substrate limitation, unlabelled IDP

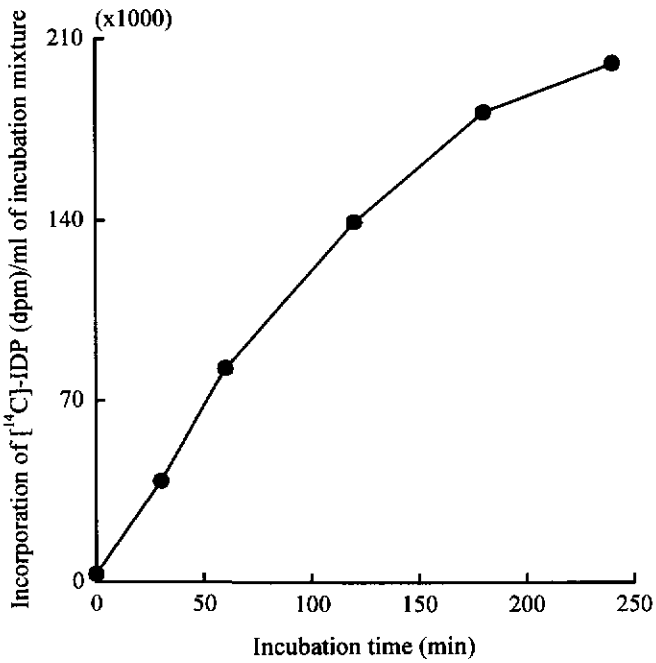


Figure 2. Conversion of [^{14}C]-IDP to rubber in reconstituted C-serum solids (2%), pH 7.5. The incubations contained [1-^{14}C]-IDP, 48 nmol (4 791 650 dpm); other additions were as in 'Basic Assay; total volume, 5 ml. A volume of 400 µl were used for purification. The incorporation values are the average of duplicate determinations.

was added. *Figure 5* shows the conversion of IDP to rubber in reconstituted C-serum (2%) as a function of IDP concentration. The optimum concentration of unlabelled IDP was found to be about 0.3 mM, which was therefore used in the final version of the assay.

Effect of WRP concentration. When WRP, rather than reconstituted C-serum, were used, it was necessary to add an initiator allylic diphosphate (in this case NDP or FDP), so that rubber synthesis could proceed at a sufficient rate, as has been observed by others[2–4]. This is

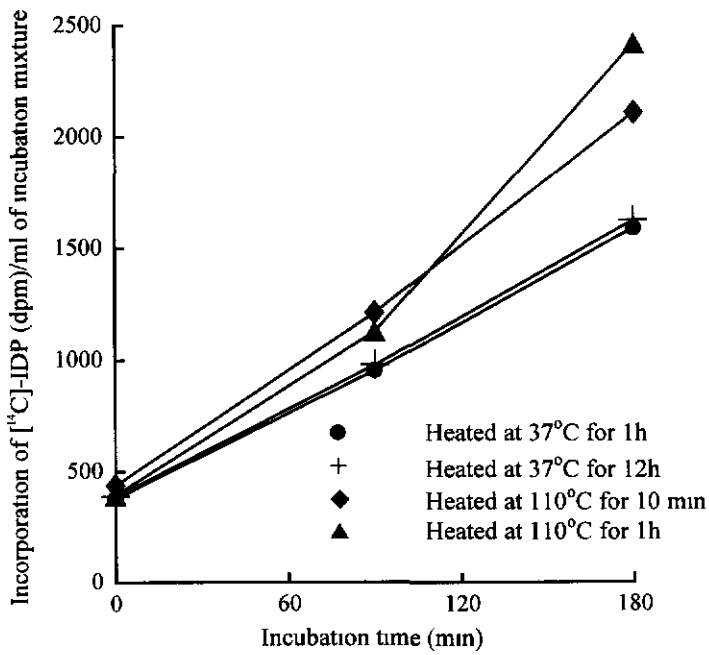


Figure 3 The effect of heating rubber trapped on gf/f membranes (Whatman) on the subsequent retention of ^{14}C-rubber synthesised in reconstituted C-serum solids (2%) The incubations contained 8 65 nmol (862 497 dpm)[1-^{14}C]-IDP, other additions were as in Basic Assay, total volume, 6 ml A volume of 200 µl were used for purification The incorporation values are the average of duplicate determinations

because the enzymes making initiators are largely removed by the gel-filtration used to isolate WRP *Figure 6* shows that [^{14}C]-IDP incorporation increases markedly with increasing WRP concentration, in the presence of 0.7 mM NDP. In the final assay, 50 µl of the concentrated WRP were used in a total volume of 200 µl. Adding more WRP would be expected to increase the incorporation of labelled IDP, but at the cost of lowering the filtration rate.

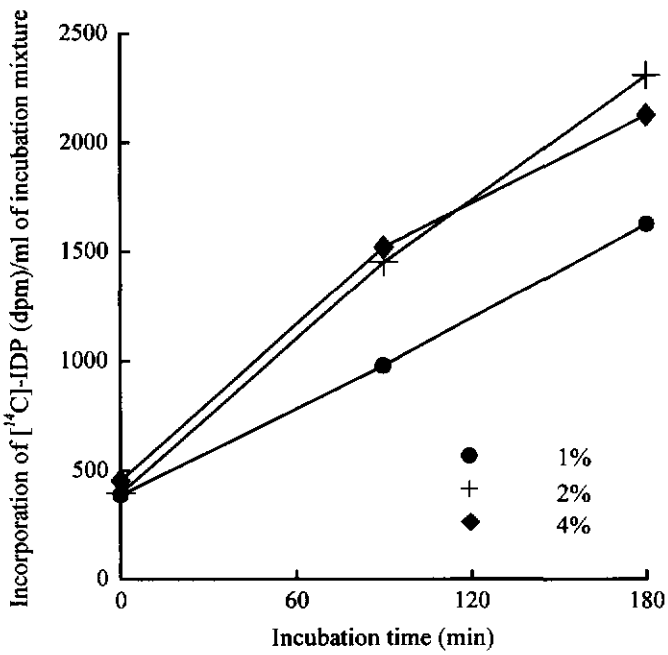


Figure 4. The effect of C-serum solids concentration on the conversion of [^{14}C]-IDP to rubber. Each incubation mixture of 200 µl contained 0.29 nmol (28 750 dpm) [1-^{14}C]-IDP and C-serum solids as shown. Other additions were as in Basic Assay. The incorporation values are the average of duplicate determinations.

Effect of dithiothreitol (DTT) concentration. The incorporation of IDP into rubber generally requires a thiol to be present[7]. In this assay, in addition to the 2-mercaptoethanol present in the WRP suspension, DTT was used. *Figure 7* shows the effect of DTT concentration on [^{14}C]-IDP incorporation, using WRP. The incorporation increased linearly up to 25 mM, and with a plateau towards 50 mM. In the final version of the assay, 50 mM DTT was used.

Effect of Mg^{2+} concentration. Rubber transferase requires a divalent metal ion for activity[1,7] and Mg^{2+} ion as $MgSO_4$, has been

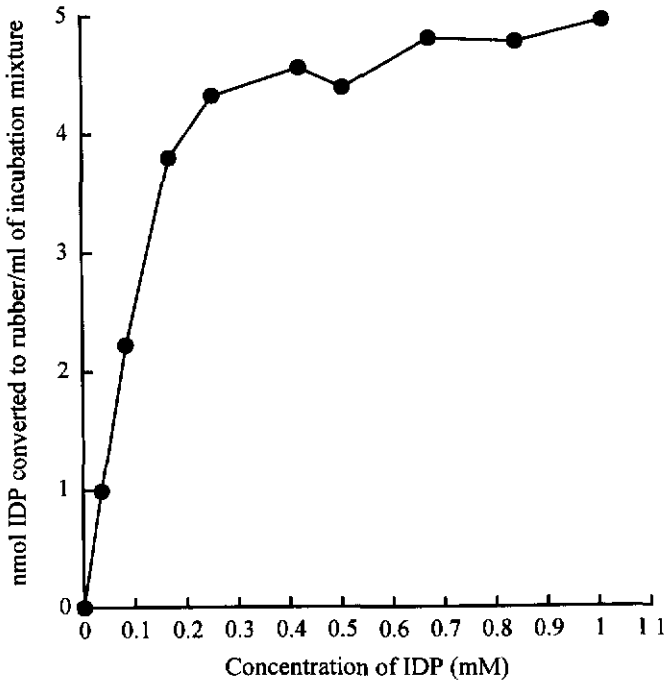


Figure 5. The effect of IDP concentration on the formation of rubber in reconstituted C-serum solids (2%). Each incubation mixture of 200 µl contained 0.29 nmol (28 750 dpm)[1-^{14}C]-IDP plus unlabelled IDP as shown; other additions were as in Basic Assay; incubation time 180 min. The incorporation values are the average of duplicate determinations.

used throughout these experiments. Using WRP, the optimum Mg^{2+} concentration was 2 mM, as shown in *Figure 8*, and therefore this concentration was used in the final assay. At higher magnesium concentrations, incorporation decreased, as found by others[1].

Effect of initiator molecules. The presence of NDP and FDP increased the incorporation of [^{14}C]-IDP in WRP by factors of approximately two-fold and three-fold, respectively [*Figures 9(a)* and *(b)*]. In the version of the assay finally adopted, NDP was

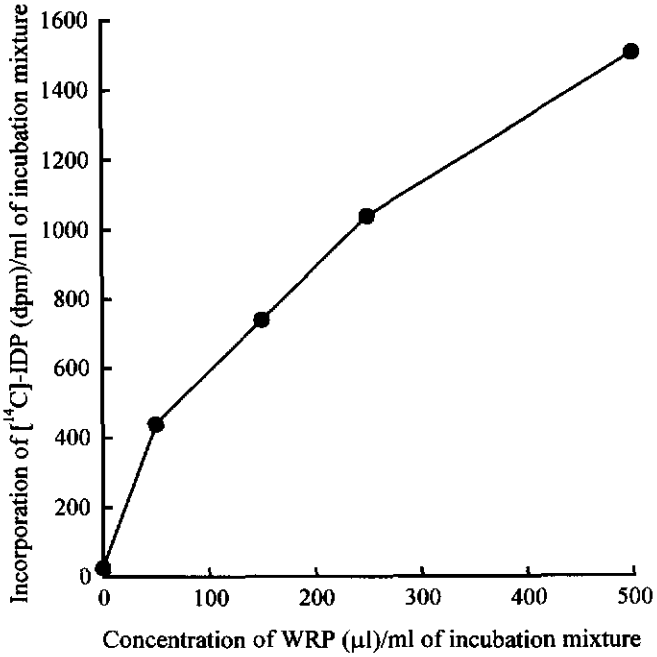


Figure 6. Conversion of [^{14}C]-IDP to rubber in a suspension of WRP of increasing concentration. Each incubation mixture of 200 µl contained 0.29 nmol (28 750 dpm)[1-^{14}C]-IDP, 0.3 mM IDP, 0.7 mM NDP and WRP as shown; other additions were as in Basic Assay (except no C-serum solids); incubation time 180 min. The incorporation values are the average of duplicate determinations.

preferred to FDP, because it was much purer by TLC analysis.

Incubation time. Figure 10 shows that the rate of conversion of IDP to rubber in WRP is linear up to at least ten hours. However, given the need for speed, a standard incubation time of three hours was ultimately used.

Effect of NaCl concentration. Since one of the reasons for developing the assay was to use it for monitoring the purification of factors

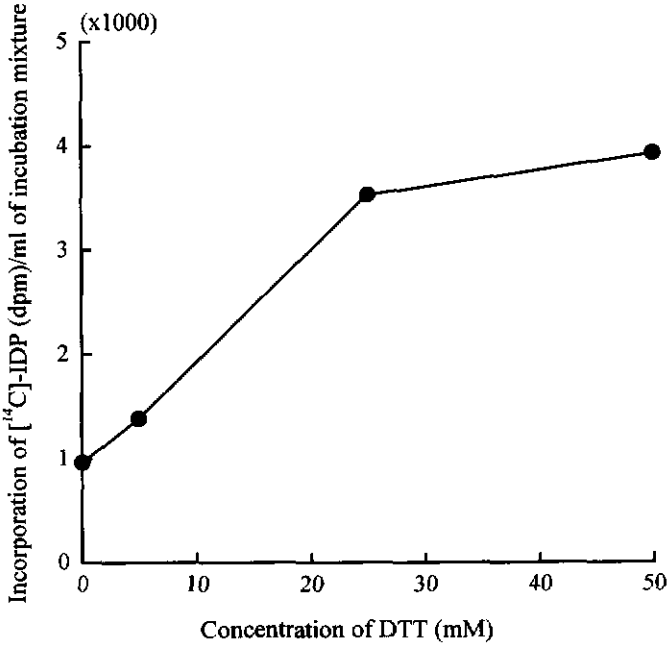


Figure 7. The effect of dithiothreitol (DTT) concentration on the conversion of [^{14}C]-IDP to rubber by WRP. Each incubation mixture of 200 µl contained 0.29 nmol (28 750 dpm)[1-^{14}C]-IDP, 50 µl WRP, 0.3 mM IDP, 0.7 mM NDP and DTT as shown; other additions were as in Basic Assay (except no C-serum solids); incubation time 180 min. The incorporation values are the average of duplicate determinations.

involved in rubber biosynthesis, the effect of NaCl, commonly used in ion-exchange chromatography, on incorporation of [^{14}C]-IDP in WRP, was investigated. NaCl was shown to have a slight stimulatory effect up to 0.1 M, but at higher concentrations became increasingly inhibitory (*Figure 11*).

Effect of phosphate concentration. For the reason given above, the effect of potassium phosphate buffer (pH 7.0) concentration was also examined. It was found that, with WRP, a 50% inhibition of IDP incorporation occurred at a phosphate concentration of 75 mM (*Figure 12*).

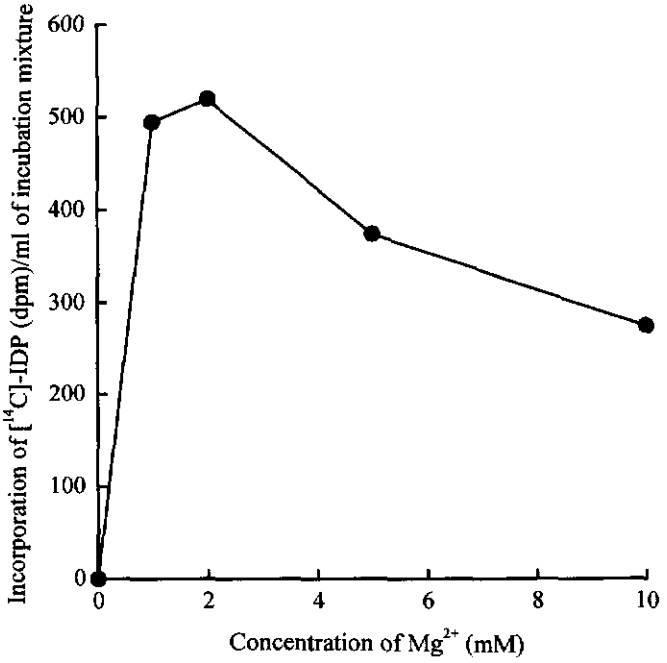


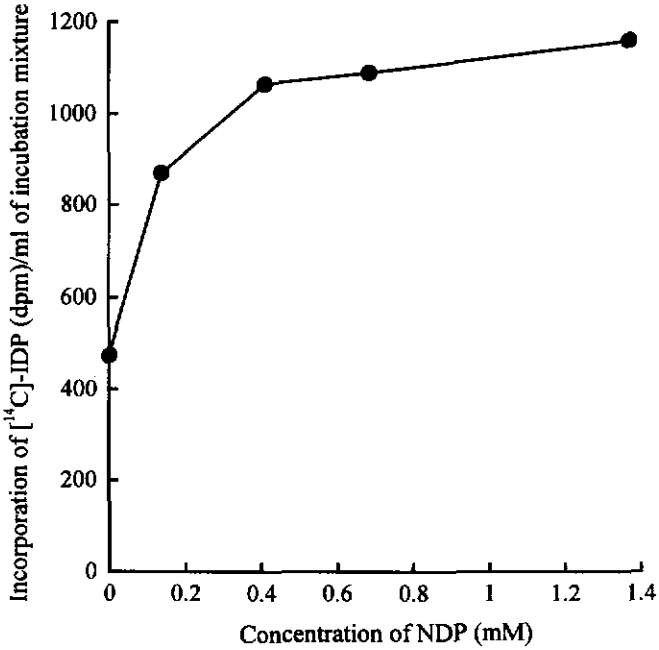
Figure 8. The effect of Mg^{2+} concentration on the conversion of [^{14}C]-IDP to rubber by WRP. Each incubation mixture of 200 µl contained 0.29 nmol (28 750 dpm)[1-^{14}C]-IDP, 50 µl WRP, 0.3 mM IDP, 0.7 mM NDP and Mg^{2+} as shown; other additions were as in Basic Assay (except no C-serum solids); incubation time 180 min. The incorporation values are the average of duplicate determinations.

Final Assay Conditions

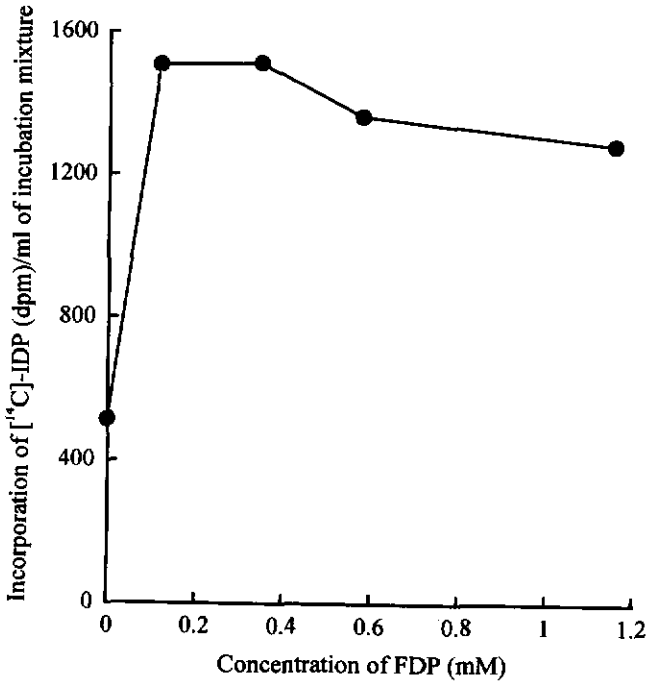
The assay finally adopted was as follows: Each 200 µl incubation mixture contained 50 µl of WRP suspension, 0.29 nmol (28,750 dpm)[^{14}C]-IDP, 0.7 mM NDP, 0.3 mM unlabelled IDP, 2 mM $MgSO_4$, 50 mM DTT and 50 mM Tris-HCl, pH 7.5.

Incubations were carried out for three hours at 25°C in the wells of a 96-well microtitre plate. The reaction was stopped by the addition

(a)



(b)



Figures 9(a) and 9(b). The effect of initiator molecule concentration on the conversion of [^{14}C]-IDP to rubber by WRP. Each incubation mixture of 200 µl contained 0.29 nmol (28 750 dpm)[1-^{14}C]-IDP, 50 µl WRP, 0.3 mM IDP and NDP or FDP as shown; other additions were as in Basic Assay (except no C-serum solids); incubation time 180 min; (a) for NDP and (b) for FDP. The incorporation values are the average of duplicate determinations.

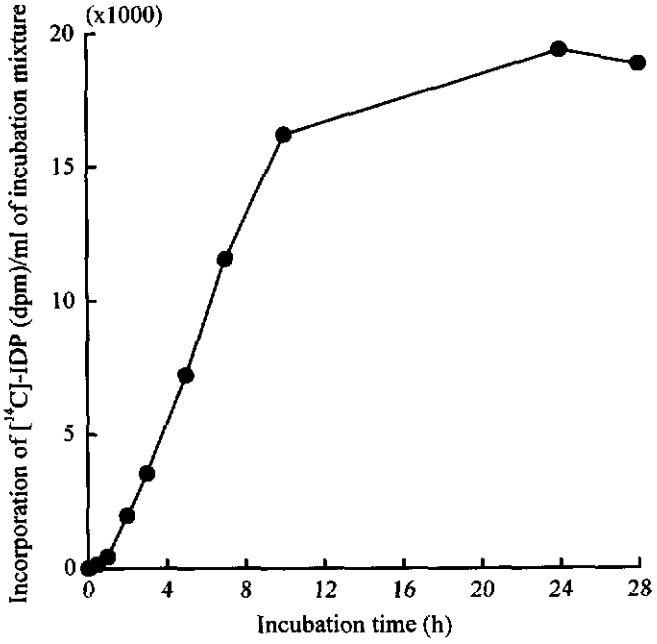


Figure 10. Conversion of [^{14}C]-IDP to rubber by WRP over an extended period, in the presence of 0.7 mM NDP. The incubations contained 7.2 nmol (718 750 dpm)[1-^{14}C]-IDP, 1250 µl WRP and 0.3 mM IDP; other additions were as in Basic Assay (except no C-serum solids); total volume 5 ml. A volume of 200 µl were used for purification. The incorporation values are the average of duplicate determinations.

of 30 µl of 0.3 M EDTA, and 10 µl of 'carrier' rubber added. 50 µl 1% Alcian Blue dye solution were then filtered through a sheet of a Whatman gf/f glass-fibre membrane on a Bio-Dot Apparatus, followed by the incubated mixtures. The membrane was disassembled from the Bio-Dot Apparatus, placed in a container, heated at 110°C for 10 min, after which it was washed once in 20 ml of 1 M HCl for 15 min, followed by thrice in 20 ml of 95% absolute ethanol for 10 min each wash. During the washings, the membranes were agitated on an orbital shaker. After drying at 110°C for about five minutes, the radioactive

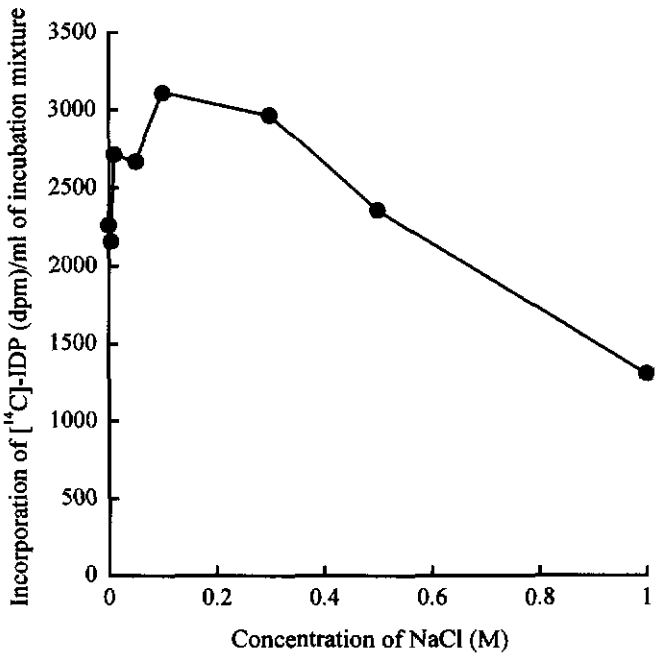


Figure 11. The effect of NaCl concentration on the conversion of [^{14}C]-IDP to rubber in WRP. Each incubation mixture of 200 µl contained 0.29 nmol (28 750 dpm)[1-^{14}C]-IDP, 50 µl WRP, 0.3 mM IDP, 0.7 mM NDP; other additions were as in Basic Assay (except no C-serum solids); incubation time 180 min. The incorporation values are the average of duplicate determinations.

areas, as indicated by the dye, were cut out, placed in scintillation vials, scintillation fluid added, and the radioactivity determined. It should be noted that the conversion of IDP to rubber is in fact higher than actually measured, because considerable [^{14}C]-rubber was lost on filtration (*Table 1*).

All incorporation measurements were carried out in duplicate. Analysis of variance, employing the Nested Designs computer programme, on 50 separate measurements shows that the method of assay is highly reproducible. The error arising from the random variation between duplicate readings

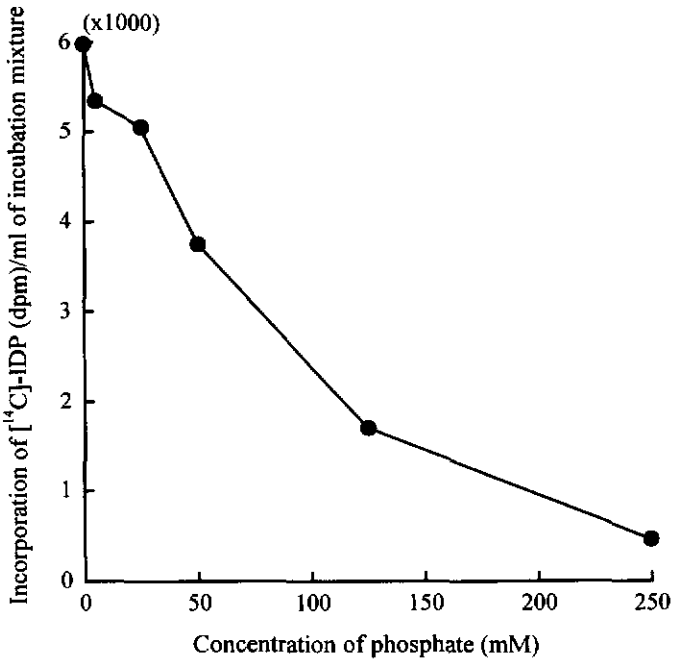


Figure 12. The effect of phosphate buffer concentration on the conversion of [^{14}C]-IDP to rubber in WRP. Each incubation mixture of 200 µl contained 0.29 nmol (28 750 dpm)[1-^{14}C]-IDP, 50 µl WRP, 0.3 mM IDP, 0.7 mM NDP and potassium phosphate buffer; pH 7.0 as shown; other additions were as in Basic Assay (except no C-serum solids); incubation time 180 min. The incorporation values are the average of duplicate determinations.

accounts for only 0.5% of the total variation observed.

Validation of Membrane-filtration Assay by Gel-filtration

Figure 13 shows the elution profiles obtained on gel-filtration of incubation mixtures containing WRP and [^{14}C]-IDP. In the run performed with the zero-time mixture, hardly any radioactivity was found in the void volumes, confirming that gel-filtration is very effective in separating rubber from IDP (with WRP, the formation of significant quantities of metabolites other than rubber, can be disregarded). Thus the method provides a good

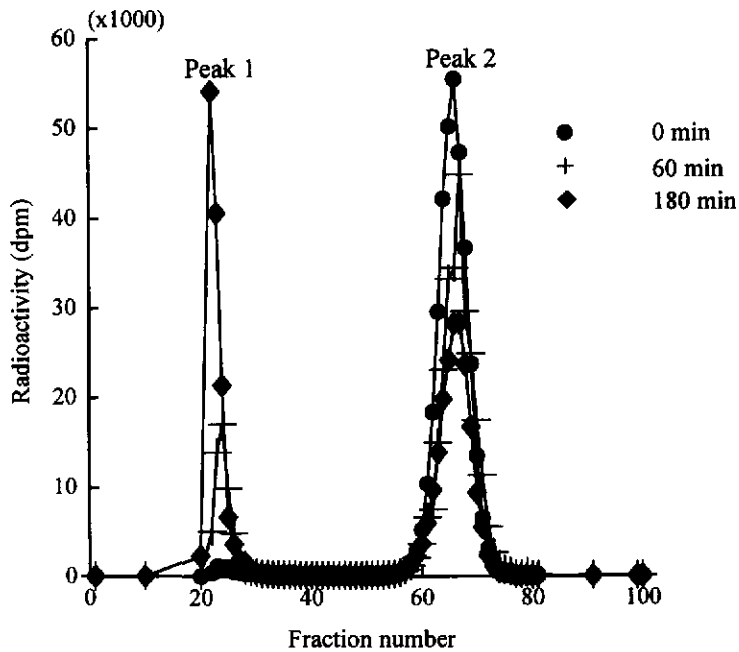


Figure 13. Assay of rubber biosynthesis by means of gel-filtration on a Sephadex G-150 *column (50 × 1.6 cm), equilibrated with 250 mM Tris-HCl buffer, pH 8.0; for details of incubation mixtures, see text. A volume of 1 ml of incubation mixture was chromatographed in each case, using the same buffer for elution; fraction volume, 1.5 ml; flow-rate, 0.5 ml per minute. The elution profiles obtained for different incubation times are shown: Peak 1: newly formed rubber; Peak 2: unreacted IDP, and possibly its low molecular weight metabolites.*

base with which to compare the membrane-filtration assay.

Table 1 shows there is a good correlation between the two methods (r=0.9, 18 experiments). So despite the fact that only about 10% (with a variation of 8.0% to 12.3%) of the rubber synthesised is measured by the membrane assay (*Table 1*), it is apparent that this method can be reliably used to compare rates of rubber synthesis under various conditions.

TABLE 1. COMPARISON OF MEMBRANE-FILTRATION AND GEL-FILTRATION ASSAYS FOR RUBBER BIOSYNTHESIS

Experiment no.	Radioactivity in rubber (dpm)	
	Membrane-filtration	Gel-filtration
1	714	8 568
2	894	9 996
3	953	11 233
4	1 296	16 200
5	1 335	16 554
6	1 674	17 585
7	1 680	16 664
8	533	5 503
9	784	7 800
10	1 077	8 750
11	584	6 891
12	972	10 150
13	1 218	10 834
14	1 002	13 126
15	1 341	12 421
16	1 047	13 192
17	1 320	13 236
18	978	10 322

Incubation mixtures were set up in duplicate for each type of analysis. They contained, in a total volume of 200 µl, 0.29 nmol (28 750 dpm)[1-^{14}C]-IDP, 50 µl WRP, 0.3 mM IDP, 0.7 mM NDP; other additions were as in Final Assay Conditions; incubation time, 180 min; the whole mixture was used for assay. Membrane-filtration was as in Final Assay Conditions, and gel-filtration as in *Figure 13*; gel-filtration was not always carried out on the day of incubation. Radioactivities are averages of the duplicate determinations.

CONCLUSION

A quick, under-five-hour assay for comparing the relative rates of rubber biosynthesis of up to 96 samples at a time, is presented here. This assay was designed for rapid-throughput of samples and this is impossible with a gel-filtration assay. The method is considerably faster than those previously described[2–4,6–8,10] Due to its simplicity, the assay is appropriate for monitoring the purification of factors involved in rubber biosynthesis in C-serum solids, and has already been successfully applied in such investigations[14–16]

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