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Biochemical Problems of Rubber Synthesis

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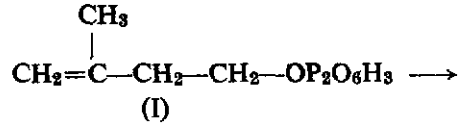
The individual steps in the synthesis of rubber from simple building blocks are now well understood, and experimental work is focussing on the physiologically important factors which regulate this process. Elucidation of the regulatory mechanism would find immediate practical application, if results suggest a means to control or stimulate rubber production in the *Hevea* plant. While it is possible that factors yet unknown, such as the spatial relationship of enzymes to building blocks and product, may play important roles in the regulatory mechanism, it is more probable that regulation of rubber biosynthesis is due to the levels and properties of enzymes already identified and partially characterised. For this reason, I may begin with a review of the experimental evidence for specific intermediates and enzymes in the pathway leading to rubber. The pathway as it is now understood is the result of accumulated work carried out vigorously and independently in many laboratories. It furnishes a necessary introduction to more recent experiments in the area of regulation and the basis for some speculation.

RUBBER—FORMULATION OF BIOGENETIC ISOPRENE RULE

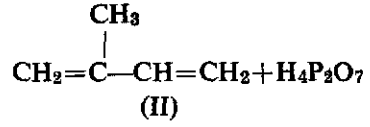
The structural analysis of rubber itself first suggested the nature of the fundamental building block and the route of biosynthesis. BOUCHARDAT (1875) proposed that, since the unsaturated hydrocarbon isoprene was generated during pyrogenic decomposition of rubber, rubber was composed of isoprene units linked together to form a polymer. This prediction was later confirmed by the experiments of HARRIS (1919) and STAUDINGER (1932). Subsequently it was found that isoprene is a constituent not only of rubber, but also of many other natural products, including the mono- and polyterpenes, the sterols and bile salts, and the carotenoids.

Rubber, however, differs from the majority of isoprenoid compounds in two respects. It has a high and variable molecular weight ranging from 100 000 to several million (SCHULZ AND MULA, 1961) and the geometrical configuration of the double bonds is exclusively *cis* (BUNN, 1942; GOLUB *et al.*, 1962). Other isoprenoid compounds have fixed molecular weights in the range of 100–1000 and, primarily, the *trans* configuration. The pattern of five-carbon sub-units apparent in both rubber and the other isoprenoids, nonetheless, suggested a common precursor and led RUZICKA (1953), on the basis of studies cited above, to formulate the biogenetic isoprene rule. The precursor turned out to be not isoprene itself, but active isoprene identified in 1958 as Δ^3-isopentenyl-pyrophosphate (I) in the laboratory of Konrad Bloch (CHAYKIN *et al.*, 1958) and in our laboratory in Munich (LYNEN *et al.*, 1958a).

This compound is simply the addition product of isoprene (II) and pyrophosphoric acid.



(I)



(II)

The studies in our laboratory which led to the discovery of isopentenyl pyrophosphate were an extension of previous work on acetate metabolism (LYNEN, 1965). Tracer studies had shown that acetate was incorporated into a large number of complex organic molecules

(*cf.* RICHARDS AND HENDRICKSON, 1964). ARREGUIN *et al.* (1951), using intact guayule seedlings and tissue cultures from this plant, demonstrated that C^{14} from carboxy-labelled acetate was also incorporated into rubber. BANDURSKI AND TEAS (1957) obtained similar results with fresh latex from *Hevea brasiliensis* and demonstrated that this material contained all the enzymes and cofactors necessary for the production of rubber from acetate. This work also provided an important cell free system for further study of rubber biosynthesis.

Simultaneously, the biosynthesis of sterols was being studied in liver and yeast extracts [review *cf.* POPJAK AND CORNFORTH (1960)]. In these studies, isopentenyl pyrophosphate was discovered and its formation from acetate described (CHAYKIN *et al.*, 1958; LYNEN *et al.*, 1958a). The investigation of rubber synthesis profitted greatly from the elucidation of the cholesterol pathway since it could be assumed that the pathways to isopentenyl pyrophosphate were identical in liver, yeast and *Hevea* latex. This assumption is now very well supported by numerous experiments which have demonstrated that the same intermediates and enzymatic activities are present in these diverse systems.

MEVALONIC ACID DISCOVERY

It should be emphasised that the discovery of mevalonic acid by Wright, Folkers and associates in 1956 (review *cf.* WAGNER AND FOLKERS, 1961) was a decisive point in both the elucidation of the cholesterol pathway and in the formulation of general pathways leading to isoprenoid compounds. Labelled mevalonic acid was incorporated both into cholesterol in liver extracts (TAVORMINA *et al.*, 1956) and into rubber in *Hevea* latex (PARK AND BONNER, 1958; KEKWICK *et al.*, 1959). The chemical formula of mevalonic acid (III) is shown in *Figure 1*. It is derived from β-hydroxy-β-methyl-glutaryl-CoA (IV) in a TPNH-linked reduction. That this reduction also occurs in *Hevea* latex was recently proved by Hepper and Audley (*cf.* ARCHER AND AUDLEY, 1967). They have shown that up to 50% of the active

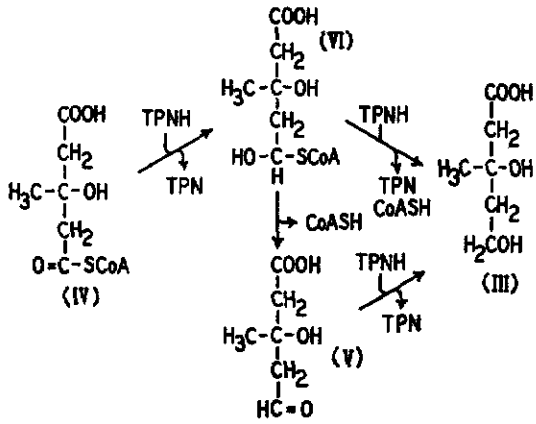


Figure 1. Possible mechanisms of mevalonic acid formation from β-hydroxy-β-methyl-glutaryl-CoA.

isomer of synthetic DL-3-C^{14}-HMG-CoA can be incorporated into rubber by incubation with latex. As expected, the incorporation of 3-C^{14}-HMG-CoA was reduced by the presence of unlabelled mevalonate, probably as the result of isotope dilution. The particular enzyme involved in the reduction of HMG-CoA in yeast extracts has been purified. Careful studies with the purified enzyme indicated mevaldic acid (V) is not an intermediate and that added mevaldic acid was only very slowly reduced (DURR AND RUDNEY, 1960; KNAPPE *et al.*, 1959). More recent experiments conducted by Dr. Rétey (unpublished) suggest that the semi-mercaptal of mevaldic acid formed with coenzyme A (VI), is much more rapidly reduced by the purified enzyme and seems to satisfy the kinetic requirements of an intermediate.

The conversion of mevalonic acid (III) to isopentenyl pyrophosphate (I) *(Figure 2)* requires two preparatory phosphorylations leading to 5-phospho- (VII) and 5-pyrophosphomevalonic acid (VIII) (*cf.* ARCHER AND AUDLEY, 1967). ATP furnishes the energy to drive these reactions forward. The subsequent step in which the biological isoprene unit is generated is chemically the most complex and interesting reaction. The enzyme involved catalyses the concerted removal of the carboxyl and the

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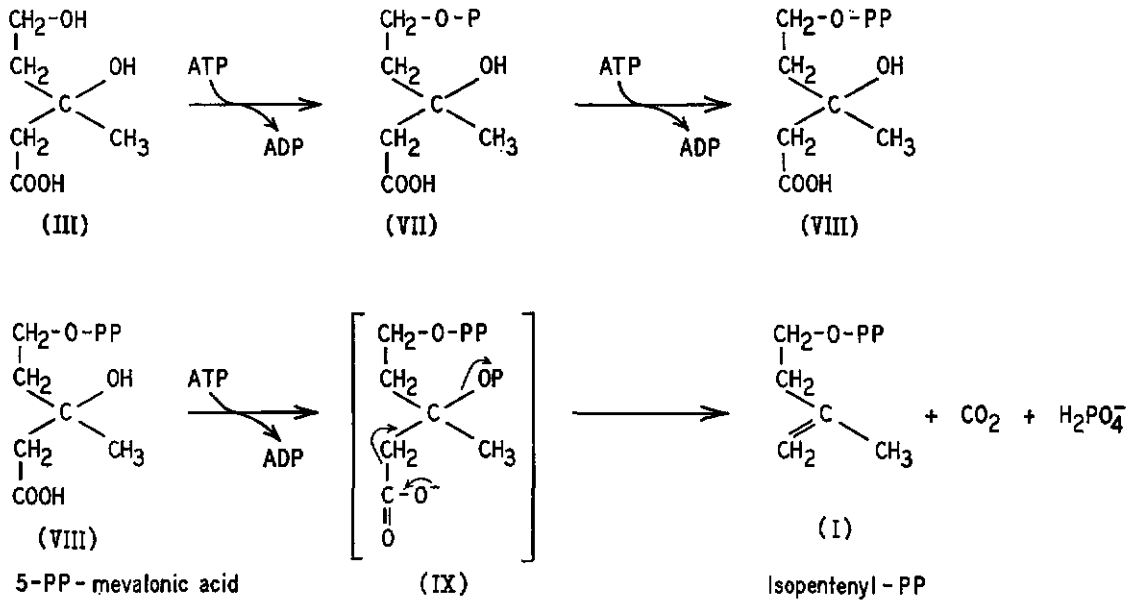


Figure 2. Mechanism of the formation of Δ^3-isopentenyl pyrophosphate from mevalonic acid.

tertiary hydroxy function from 5-pyrophosphomevalonic acid. Data of Bloch and his associates (LINDBERG *et al.*, 1962) obtained with O^{18} suggest that 3-phosphomevalonate-5-pyrophosphate (IX) is a transitory intermediate, ATP again serving as phosphorylating agent for the tertiary hydroxyl group in a reaction which facilitates its elimination.

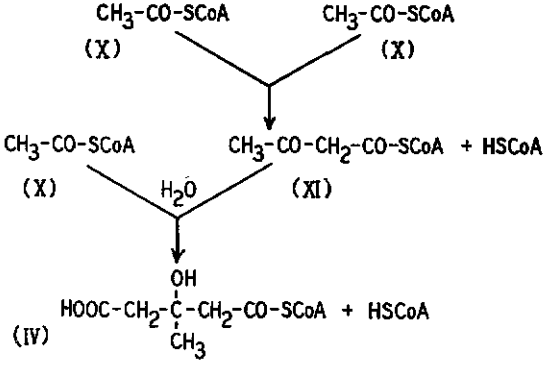


Figure 3. Mechanism of the formation of β-hydroxy-β-methyl-glutaryl-CoA from acetyl-CoA.

In reactions leading to β-hydroxy-β-methyl-glutaryl-CoA *(Figure 3)* the carbon skeleton of the C_6-precursors of isopentenyl pyrophosphate is assembled by condensation of three acetate units. Complete understanding of these reactions depends on the realisation that acetyl-CoA (X), the high energy form of acetate required, is activated both at the carbonyl and at the methyl group (JAENICKE AND LYNEN, 1960). In the condensation of two acetyl-CoA molecules (X) to form acetoacetyl-CoA (XI) both activated groups are involved. One molecule of acetyl-CoA is the nucleophilic agent and acetate acceptor, the other is the electrophilic acetyl donor. The formation of acetoacetyl-CoA (XI) is reversible and the equilibrium constant of the thiolase reaction at pH 7:

$$K_{eq}=\frac{[\text{acetoacetyl-CoA}]\,[\text{CoA}]}{[\text{acetyl-CoA}]^2}=1.6\times10^{-5}$$

(JAENICKE AND LYNEN, 1960)

indicates that very little acetoacetyl-CoA is formed from acetyl-CoA by the isolated enzyme under equilibrium conditions. Never-

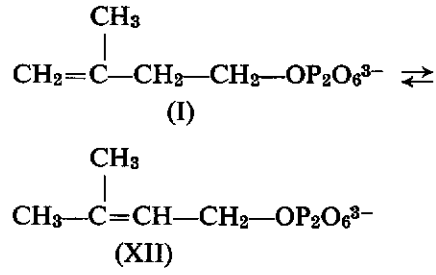
theless, net synthesis of acetoacetyl-CoA at the rate necessary for formation of isoprenoid compounds, can be achieved if the product is rapidly removed from the unfavourable equilibrium by further condensation with another molecule of acetyl-CoA. This second condensation resulting in β-hydroxy-β-methyl glutaryl-CoA (HMG-CoA, IV) has been demonstrated experimentally. The reaction has the physiologically important feature of irreversibility. At least, it has not been possible to demonstrate any cleavage of HMG-CoA even under conditions which should favour this (LYNEN *et al.*, 1958b; RUDNEY AND FERGUSON, 1959). Thus the second condensation imposes a dynamic equilibrium on the thiolase reaction which results in the accumulation of HMG-CoA.

There are several reports that malonyl-CoA, formed from acetyl-CoA and CO_2 in an ATP requiring reaction, is involved in the synthesis of HMG-CoA (*cf.* ARCHER AND AUDLEY, 1967). From the thermodynamic point of view, however, there is no need for the expenditure of ATP in the formation of HMG-CoA from three units of acetyl-CoA. In addition there is good experimental evidence that a pathway to HMG-CoA involving malonyl-CoA, if existing at all under physiological conditions, plays only a relatively minor role compared to the pathway involving the two condensations mentioned above. This problem will be discussed more fully later.

CONDENSATION REACTIONS— CLUE TO POLYMERISATION

Most relevant to the synthesis of rubber are the condensation reactions following synthesis of isopentenyl pyrophosphate (I). Again, as in the case of acetyl-CoA, the bifunctional nature of the active isoprene building block is a key to the polymerisation reactions. In the case of isopentenyl pyrophosphate this bifunctionality resides in the nucleophilic reactivity of the Δ^3-double bond and the potential electrophilic character of the pyrophosphate ester.

Condensation is initiated by the isomerisation of isopentenyl pyrophosphate (I) to dimethylallyl pyrophosphate (XII) by a shift of the double bond (LYNEN *et al.*, 1959):



With the formation of dimethylallyl pyrophosphate the electrophilic reactivity of the precursor isopentenyl pyrophosphate is fully realised. Ionisation of the carbon–oxygen bond of the dimethylallyl pyrophosphate creates a cationic centre which attacks the electrons available in the exomethylene group of isopentenyl pyrophosphate. The elimination of a proton leads to the first condensation product, an allyl pyrophosphate containing ten carbon atoms *(Figure 4)*. This homologous allyl pyrophosphate by aquisition of another isopentenyl pyrophosphate is converted into a C_{15}-compound. Repetition of this process eventually leads to high molecular weight polymers or rubber.

The configuration of the double bonds in a polyisoprenoid product is determined by enzymes which can differentiate between the two

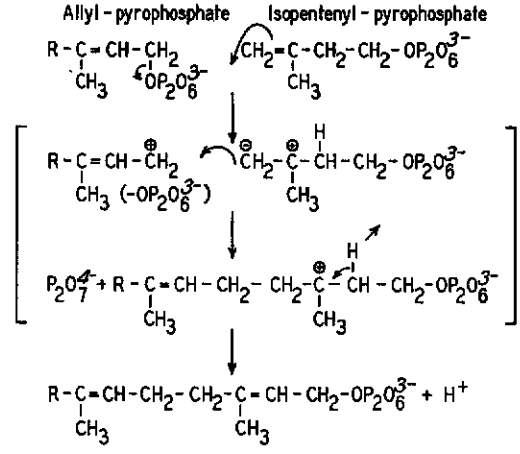


Figure 4. Hypothetical mechanism of the synthesis of the isoprenoid carbon chain.

chemically equivalent hydrogens at C-2 in isopentenyl pyrophosphate. Experimental evidence that an enzyme involved in rubber biosynthesis is capable of this feat was obtained in a very elegant manner by Archer and coworkers in collaboration with Cornforth and Popják (ARCHER *et al., 1966*). *Starting with* [2-C^{14}-(4R)-4-$_1H^3$] or [2-C^{14}-(4S)-4-$_1H^3$] mevalonic acid—tritium in the C-4 position—they were able to generate [4-C^{14}-(2S)-2-$_1H^3$) or [4-C^{14}-(2R)-2-$_1H^3$] isopentenyl-pyrophosphate respectively. Each of these compounds was tested in a latex preparation from *Hevea brasiliensis* capable of synthesising rubber. This same preparation was also capable of diverting some mevalonate into farnesol. The H^3/C^{14} ratio in rubber synthesised from (2R)-isopentenyl pyrophosphate was the same as that found in the original [2-C^{14}-(4S)-4-$_1H^3$] acid, but rubber derived from (2 S)-isopentenyl pyrophosphate contained practically no tritium. In the case of farnesol the opposite occurs. Rubber contains double bonds in the *cis* configuration; farnesol contains double bonds in the *trans* configuration as determined by independent physical methods.

From the stereochemical work of POPJAK AND CORNFORTH (1966) it must be concluded that no free carbonium ions are generated from the allylic pyrophosphates and that condensation is accompanied by the concerted removal of a proton.

In connection with these studies, it was found that, during chain growth, inversion of configuration at C-1 of the allylic pyrophosphate occurs. To explain this observation, Cornforth and Popják suggested an S_N2 mechanism for the condensation in which the pyrophosphate ion is displaced in synchrony with attachment of a new isopentenyl unit. In the absence of neighbouring group effects, such reactions occur with inversion of configuration. The detailed chemical mechanism of rubber synthesis, as depicted in *Figure 5* forces one to postulate an electron-donating group X that is temporarily attached at C-3. The chemical nature of X cannot be specified. It may be a group on the enzyme, or possibly the pyrophosphate anion of isopentenyl pyrophosphate

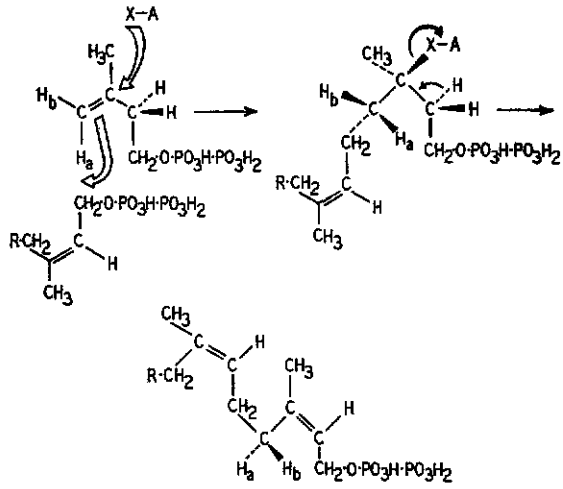


Figure 5. Stereochemistry of rubber synthesis from isopentenyl pyrophosphate.

itself as suggested by JOHNSON AND BELL (1960).

Experimental efforts in our laboratory centred round the demonstration and characterisation of the enzyme that catalyses the polymerisation of isopentenyl pyrophosphate to form squalene. Shortly after isopentenyl pyrophosphate had been discovered and Eggerer (EGGERER AND LYNEN, 1960) in our laboratory had achieved the chemical synthesis of this compound, Henning (HENNING *et al.*, 1961) proved that freshly tapped latex from *Hevea brasiliensis* catalysed the incorporation of C^{14}-labelled isopentenyl pyrophosphates into rubber. In these preliminary experiments, the methods of PARK AND BONNER (1958) were used. After incubation of latex with the substrate labelled at C-4, the rubber was isolated and oxidised with ozone *(Figure 6)*. Authentic samples of laevulinic acid and acetone were added to the ozonolysis products and the crystalline 2,4-dinitrophenyldrazones prepared and isolated. The hydrazone of laevulinic acid contained radioactive carbon from 4-C^{14}-isopentenyl-pyrophosphate, but the acetone fraction contained none *(Table 1)*. This indicated that the ratio of terminal C_5-units to internal C_5-units in rubber is extremely small.

The nature of the end-product rubber poses some formidable spatial problems for the poly-

merising enzyme. *Hevea* latex is a colloidal suspension of rubber and other particles in an aqueous medium. Freshly tapped latex can be divided into three distinct fractions by centrifugation: a light rubbery phase of creamy appearance, an aqueous phase generally called the latex serum, and a heavier fraction composed of membraneous particles called lutoids. As first shown by Archer and his associates (ARCHER *et al.*, 1963) the conversion of isopentenyl pyrophosphate to rubber takes place on the surface of existing rubber particles which are essential for the *in vitro* formation of polyisoprene. According to these authors the incorporation of C^{14}-labelled isopentenyl pyrophosphate is predominantly a chain extension process involving already existing rubber chains which presumably carry allyl pyrophosphate end-groups (ARCHER AND AUDLEY, 1967).

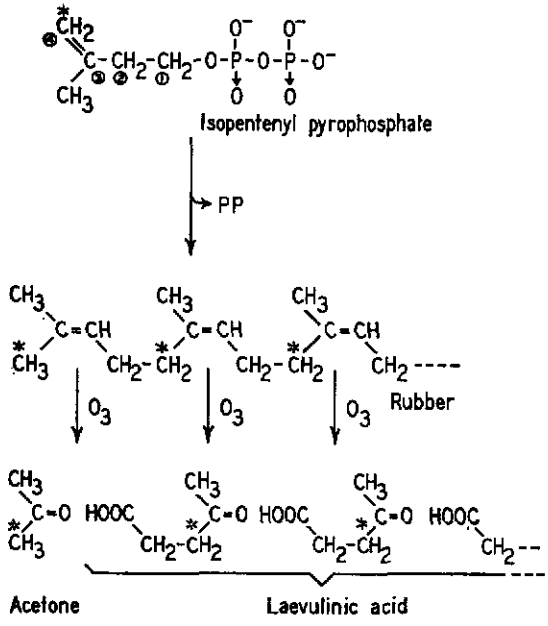


Figure 6. Scheme of the incorporation of 4-C^{14}-isopentenyl pyrophosphate into rubber and its ozonolysis.

TABLE 1. OZONISATION OF RUBBER FORMED FROM 4-C^{14}-ISOPENTENYL PYROPHOSPHATE*

Fraction	Radioactivity (c.p.m.)
Acetone	0
Laevulinic acid	13 600

246 mg of latex, 6 μmoles of MgK_2-ethylenediamine tetraacetate, 1 μmole of $MgCl_2$ and 0.23 μmoles of K_3-4-C^{14}-isopentenyl pyrophosphate (1.7×10^5 c.p.m.) were incubated for 4 hours at 26°C. The crude rubber was isolated and oxidised with ozone. After addition of carrier material the 2,4-dinitrophenylhydrazones of acetone and laevulinic acid were isolated and counted.

*See HENNING *et al.* (1961)

RUBBER SYNTHESIS ON PARTICLE SURFACE ONLY

Experiments in our laboratory supported the conclusion of the British biochemists that rubber synthesis takes place on the surface of rubber particles. However, chain initiation was also observed. These studies were performed in collaboration with Dr. Berndt, Dr. Dick, Mrs. Hopper and Dr. Nordwig (LYNEN, 1967). With the generous support of Professor Milanez, then director of the famous botanical garden in Rio de Janeiro, one of the *Hevea brasiliensis* trees in the garden was put at our laboratory's disposal. Dr. Raoul Machado expertly tapped the tree, collected the latex, added cysteine and potassium bicarbonate as protective agents and shipped the material frozen in dry ice by air. In this way the enzymatically active material arrived in Munich no later than 24 hours after collection in Rio de Janeiro.

In order to measure polymerase activity the incorporation of synthetic 1-C^{14}-isopentenyl pyrophosphate into rubber was determined. As can be seen in *Figure 7*, the assay was reliable since radioactivity incorporated into rubber was proportional to the amount of latex added *(Figure 7)* and to the time of incubation up to two hours. The enzyme has a pH optimum near 8 (LYNEN, 1963) and a requirement for magnesium ions *(Figure 8)*. Maximum incorporation was observed at 2×10^{-3} M magnesium. Higher concentrations inhibited polymerisation as was also reported by Archer (ARCHER AND AUDLEY, 1967).

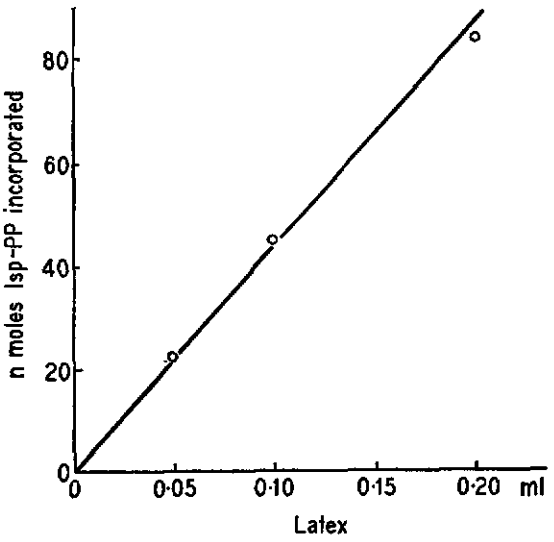


Figure 7. Assay of the polymerising enzyme in latex.

The reaction mixture contained in a total volume of 0.52 ml: 100 µmoles of TRIS-buffer, pH 8; 5 µmoles of MgK_2-ethylenediamine tetraacetate; 1 µmole of $MgCl_2$; 2 µmoles of cysteine, 0.32 µmole of 1-C^{14}-isopentenyl pyrophosphate (54 300 c.p.m.) and latex as indicated on the abscissa. After incubation for 30 minutes at 27°C the reaction was stopped by the stepwise addition of 0.1 ml of 0.1 M K_4-ethylenediamine tetraacetate and 4 ml of 2 per cent trichloroacetic acid in methanol. After centrifugation the rubber present in the precipitate was dissolved in toluene. The toluene solution was repeatedly washed with 2.5 N NaOH and then counted.

This inhibition was not observed if magnesium ions were added in the form of the complex with ethylenediamine tetraacetate (MgK_2-EDTA). The presence of SH-group(s) in the active center of the enzyme was indicated by the observation that sulphydryl blocking agents such as mercuric salts, iodoacetamide or N-ethyl maleimide inhibit the incorporation of isopentenyl pyrophosphate into rubber. Furthermore it was found that whole latex and especially the creamy layer containing rubber particles showed very little activity unless cysteine or other sulphydryl compounds had been added. This is illustrated in *Table 2.*

In order to stabilise the emulsion of rubber particles in the creamy layer 1% serum albumin and 1% Tween 20 were routinely added. In the presence of these surface-active agents, latex shows only 50–70% of its original activity (compare Experiment 1 and 2 of *Table 2*). On the other hand, these agents make it possible to isolate the creamy layer repeatedly by centrifugation and to resuspend it without coagulation. In this way the rubber particles can be extensively washed and all soluble enzymes removed. After treatment the polymerase was still present on the rubber particles, but isopentenyl pyrophosphate isomerase had been removed completely. Therefore the incorporation of 1-C^{14}-isopentenyl pyrophosphate into rubber became strictly dependent on

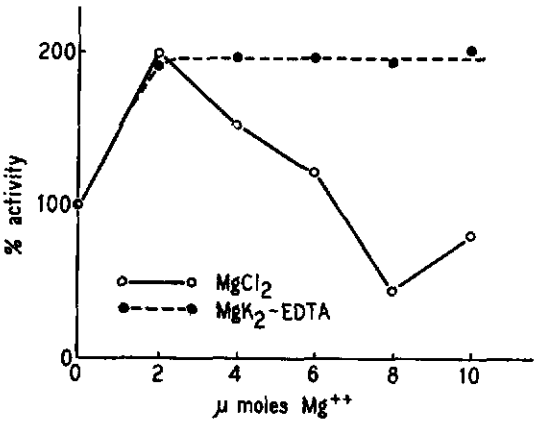


Figure 8. Effect of magnesium concentration on rubber synthesis.

The reaction mixture contained in a total volume of 0.5 ml: 100 µmoles of TRIS-buffer, pH 8; 0.2 µmole of cysteine; 0.05 µmole of dimethylallyl pyrophosphate; 0.26 µmole of 1-C^{14}-isopentenyl pyrophosphate (44 500 c.p.m.) 0.2 ml of latex and either $MgCl_2$ or MgK_2-EDTA (ethylenediamine tetraacetate) as indicated on the abscissa. After incubation for 90 minutes at 25°C the reaction was stopped and the radioactivity incorporated into the rubber was determined. For experimental details, see legend of Figure 7.

TABLE 2. PARTICIPATION OF SULPHYDRYL GROUPS IN ACTIVE CENTRE OF POLYMERISING ENZYME

Substance (0.1 ml)	Cysteine (μmoles)	Iodo-acetamide (μmoles)	C^{14}-iso-pentenyl-PP incorporated (μmoles)
Latex	5	–	57.6
Latex+Tween +serum albumin	5	–	30.6
Latex+serum albumin	–	–	8.3
Upper phase (creamy layer)	5	–	20.7
	–	–	0.9
	–	0.1	0.0
	–	0.01	0.5

The reaction mixture contained in a total volume of 0.5 ml: 100 μmoles of TRIS-buffer, pH 8.0; 2 μmoles of $MgCl_2$; 40 μmoles of MgK_2-ethylenediamine tetra-acetate; 0.1 μmole of dimethylallyl pyrophosphate; 0.17 μmoles of 1-C^{14}-isopentenyl pyrophosphate (54 000 c.p.m.); cysteine and iodoacetamide as indicated and 0.1 ml either of latex, of latex containing 1 per cent Tween 20 and (or) 1 per cent serum albumin, or of the upper phase of latex after centrifugation.

the addition of dimethylallyl pyrophosphate *(Table 3)*. As mentioned above, the isomerase plays a crucial role in the reaction sequence of polyisoprenoid synthesis. It catalyses the isomerisation of isopentenyl pyrophosphate to the allylic derivative and thus triggers chain initiation. Once chain growth has started it can continue without renewed isomerisation and is limited only by the specificity of the participating enzymes.

From the relationship of the rate of C^{14}-isopentenyl-pyrophosphate incorporation into rubber and the 'primer' concentration *(Figure 9)*, a Michaelis constant of 1.3×10^{-5} Molar for the polymerase activity with respect to dimethylallyl pyrophosphate was found. At this concentration the polymerase was half saturated with substrate. The Michaelis constant for isopentenyl pyrophosphate was found to be 1.2×10^{-4} Molar.

The enzymatically formed product was identified as high molecular weight rubber by analytical ultracentrifugation in cyclohexane. Only one high-molecular weight component which sedimented after about 8 hours centrifugation was observed *(Figure 10)*. The distribution of radioactivity after sedimentation is shown in *Figure 11*. All radioactivity was found in the 'bottom fraction'. This result excludes the possibility that the radioactive product formed from isopentenyl pyrophosphate was of low molecular weight and different from rubber. Furthermore, when the radioactive product was chromatographed on paraffinated paper with acetic acid-benzene (9:1) as solvent, all radioactivity remained at the origin whereas low molecular weight terpenes like squalene, farnesol or geraniol-linalool, when added in carrier amounts, moved away.

TABLE 3. REQUIREMENTS OF DIMETHYLALLYL PYROPHOSPHATE FOR RUBBER SYNTHESIS IN UPPER PHASE (CREAMY LAYER) OF LATEX

Experiment	Latex containing 1% Tween 20 and 1% serum albumin	Upper phase	
		with DMA-PP	without DMA-PP
	C^{14}-isopentenyl-PP incorporated (μmoles)		
1	205	89	0
2	87.5	47	3
3	40	24.6	0
4	42.5	44.5	3.5
5	30.6	20.7	0.5

The reaction mixture contained in a total volume of 0.5 ml: 100 μmoles of TRIS-buffer, pH 8.0; 20 μmoles of MgK_2-ethylenediamine tetraacetate; 2 μmoles of $MgCl_2$; 5 μmoles of cycteine; 0.17 μmole of 1-C^{14}-isopentenyl pyrophosphate (45 000 c.p.m.); 0.1 μmole of dimethylallyl pyrophosphate (DMA-PP) if indicated; and 0.1 ml latex containing 1 per cent Tween 20 and 1 per cent serum albumin or 0.1 ml of upper phase, prepared from this latex. After incubation for 60 or 90 minutes at 26°C the rubber was isolated—see legend of *Figure 7*—and counted.

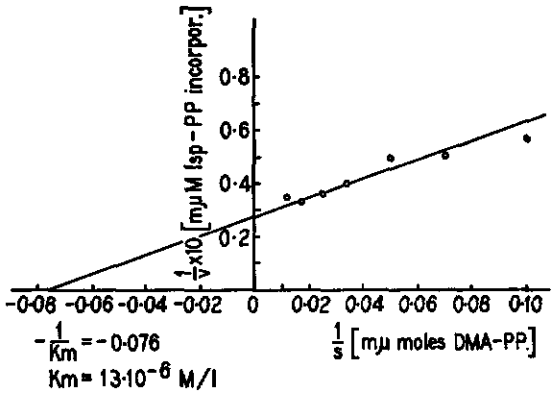


Figure 9. Determination of the Michaelis constant of the polymerising enzyme for dimethylallyl pyrophosphate.

A 0.8 ml reaction mixture contained (in μmoles): TRIS buffer, pH 8.0, 100; MgK_2-ethylenediamine tetraacetate, 20; $MgCl_2$ 2; cysteine, 5; 1-C^{14}-isopentenyl pyrophosphate (2.1×10^5 c.p.m./μmole), 0.17; 0.1 ml of the resuspended creamy upper phase of centrifuged Hevea *latex; and varying concentrations of dimethylallyl pyrophosphate. In this* Figure, *the reciprocal value of the rate of synthesis (1/v) is plotted against the reciprocal value of the dimethylallyl pyrophosphate concentration (1/s).*

The requirement of dimethylallyl pyrophosphate for 1-C^{14}-isopentenyl pyrophosphate and the failure to detect low molecular weight polymers, such as squalene or farnesol, suggest that chain initiation as well as chain elongation occurs in the cell-free system. The conclusion of ARCHER AND AUDLEY (1967) that only chain elongation occurs was prompted undoubtedly by difficulties they encountered with contaminating enzymes, which made it impossible to demonstrate a requirement for dimethylallyl pyrophosphate.

The site of polymerase activity at the interphase between serum and rubber particles appears to facilitate the production of rubber by a system, in which the hydrophilic substrates dimethylallyl pyrophosphate and isopentenyl pyrophosphate are converted into a lipophilic end-product. In *Figure 12* a hypothetical scheme outlining the events at the interphase is given. It was assumed that the growing hydrocarbon chain of rubber diffuses into the interior of the rubber droplet and that the hydrophilic pyrophosphate end-group remains in the serum phase where it can interact with isopentenyl pyrophosphate bound to the active site of the polymerase. In this way deposition of the water insoluble rubber molecule on the surface of the enzyme and its subsequent inhibition is avoided. The association between polymerase and rubber particles appears to be rather stable. In contrast to the report from ARCHER AND AUDLEY (1967) repeated attempts in this laboratory to dissociate polymerase activity from the hydrocarbon phase were without success. In this respect the polymerase differs from the other enzymes involved in rubber synthesis and responsible for the formation of isopentenyl pyrophosphate from acetate. The soluble enzymes were all found in the serum fraction obtained by centrifugation of latex. This appears to be economical from the physiological point of view since isopentenyl pyrophosphate is also a precursor of the sterols and carotenoids formed by other structural elements of latex.

The reaction sequence leading from acetate to rubber is summarised in *Figure 13*. The sequence can be divided into three sections. In the first section, the carbon skeleton of mevalonic acid is synthesised from three molecules of acetyl-CoA. The energy required for this process is derived from three thioester bonds, which are cleaved to regenerate free coenzyme A, and the two TPNH molecules, which become oxidised. In the second section the conversion of mevalonic acid to isopentenyl pyrophosphate, the building unit of the polyisoprenoids, is achieved. Activation of mevalonic acid requires three moles of ATP and a decarboxylation reaction. For the synthesis of the polyisoprenoid carbon chain in the last section, no additional source of energy is required, since sufficient energy has been made available by the previous expenditure of ATP. Pyrophosphate is a good leaving group in the ensuing S_N2 reaction and its subsequent hydrolysis to orthophosphate makes the condensation reaction irreversible. Pyrophosphatase is ubiquitous in all living systems and has been demonstrated in latex also.

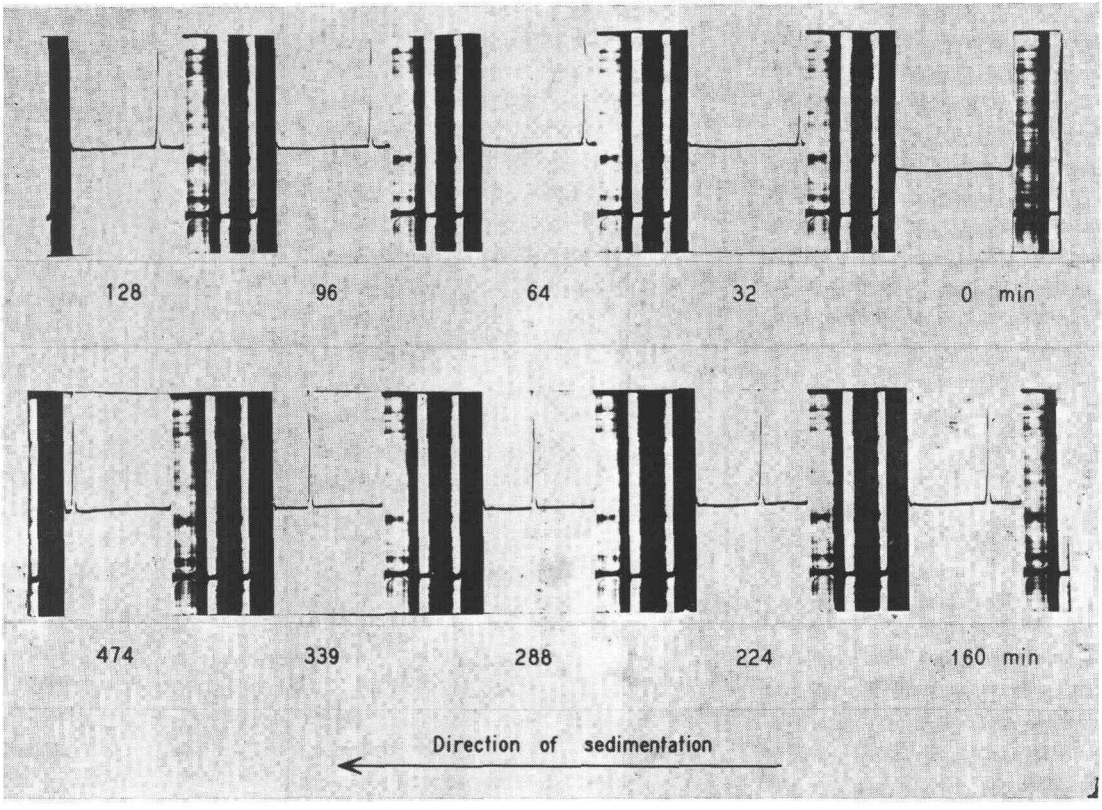


Figure 10. Schlieren-patterns of the sedimentation of crude rubber, in the analytical ultracentrifuge. Crude rubber, precipitated with methanol, was dissolved in cyclohexane to a concentration of 0.26 per cent and was centrifuged at 180 000 g. The numbers given are the time intervals after starting the ultracentrifugation.

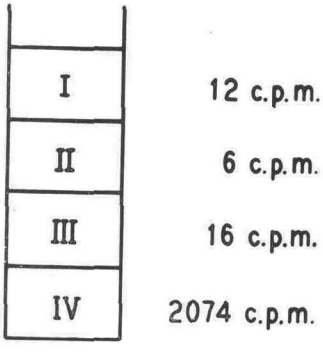


Figure 11. Distribution of radioactive material in the ultracentrifuge cell.

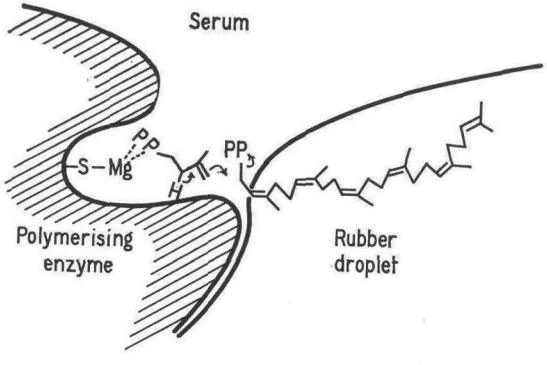


Figure 12. Hypothetical scheme outlining the mechanism of the polymerising enzyme at the interphase between the serum and the rubber droplet.

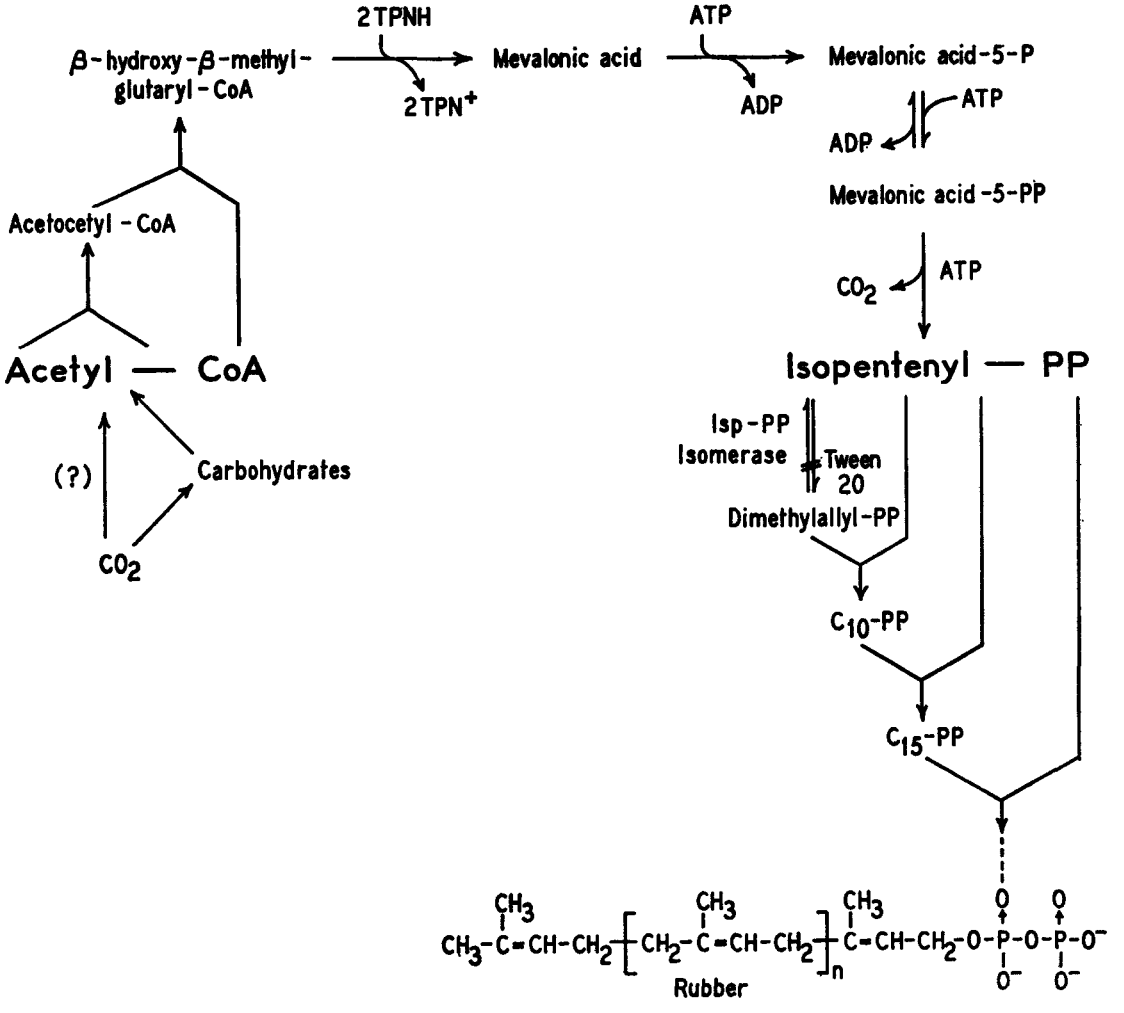


Figure 13. The route of the biosynthesis of rubber.

If one surveys the reactions of rubber synthesis, one realises that the process requires three components: acetyl-CoA as building block, TPNH as reducing agent and ATP as an energy source. All three components are generated by the degradation of carbohydrates *(Figure 14)*. This clearly suggests that *in situ* carbohydrate metabolism may become a limiting factor in rubber biosynthesis. There is good evidence that latex can metabolise sugars. D'AUZAC (1964) found that endogenous sugars gradually disappear in tapped latex. When uniformly labelled glucose is used as substrate, among the labelled products are ethanol, lactate, and Krebs-cycle acids, notably citrate and malate. Furthermore, Bealing (*cf.* ARCHER AND AUDLEY, 1967) has obtained evidence for the presence of all the enzymes of the Embden-Meyerhof pathway in latex. There can be no doubt that glycolysis is one of the main sources of ATP required for rubber synthesis. For this reason the studies on regulation of carbo-

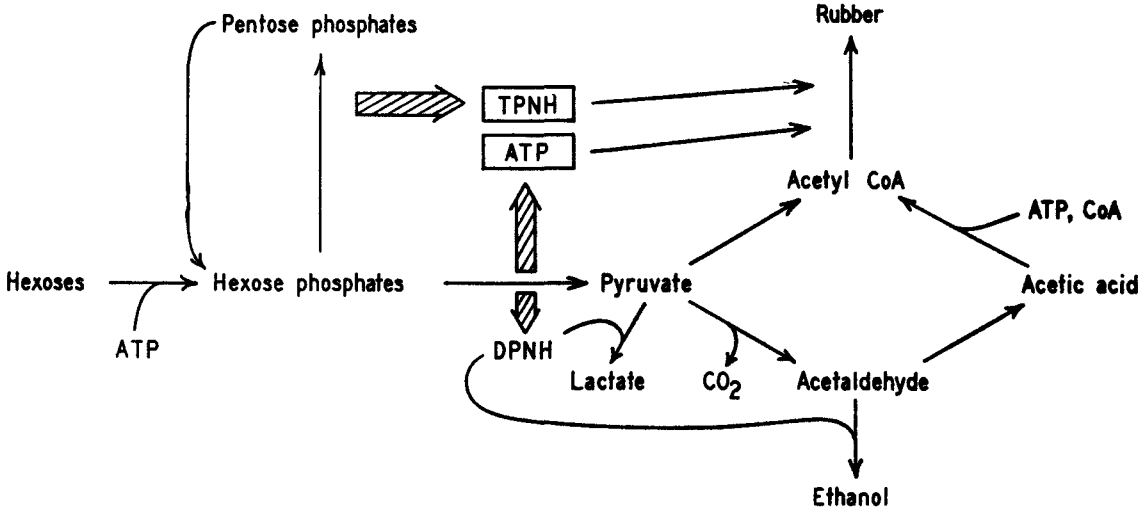


Figure 14. The interrelations between carbohydrate degradation and rubber synthesis.

hydrate metabolism as performed by d'Auzac and discussed later in this session deserve special attention.

The large portion of the TPNH required for rubber synthesis is generated by the hexose-monophosphate 'shunt', a pathway diverging from the glycolytic pathway at the level of glucose-6-phosphate *(Figure 14)*. Glucose-6-phosphate is oxidised by TPN in two subsequent steps with formation of TPNH, CO_2 and ribulose-5-phosphate. The carbon skeleton of the latter is then anaerobically rearranged and, as a result, five molecules of hexosephosphates are regenerated from six molecules ot pentose phosphates. Bealing (*cf.* ARCHER AND AUDLEY, 1967) as well as ARREGUIN AND ROCK (1967) have obtained evidence for the presence of all the enzymes of the hexose monophosphate 'shunt' in latex. RIBAILLIER *et al.* (1964) have also demonstrated that added TPNH stimulates the conversion of acetate to rubber. It can be predicted that *in situ* the TPNH concentration of latex will be high if glucose-6-phosphate is being diverted through the hexose-monophosphate 'shunt' instead of being used by the Embden-Meyerhof pathway. Attention should therefore be directed toward a detailed study of the enzyme phosphofructokinase and its regulation because it determines the pathway of sugar degradation.

Information about the origin of acetyl-CoA in latex is rather scanty *(Figure 14)*. Most workers (BANDURSKI AND TEAS, 1957; HARRIS AND KEKWICK, 1961; D'AUZAC, 1965) have found in experiments with tapped latex that the incorporation of acetate into rubber is low, and that the incorporation of pyruvate is even lower. These results are quite unexpected, since pyruvate in other polyisoprenoid synthesising systems was the more satisfactory *in vitro* precursor. As pointed out by ARCHER AND AUDLEY (1967), there are several possible explanations for the situation as it exists in *Hevea* latex. The mitochondrial population of tapped latex appears to be low compared to the population in plant cell, implying a reduction of acetyl-CoA generation in latex by pyruvate oxidase. In addition, acetyl-CoA generated in mitochondria may not be readily available for extramitochondrial syntheses. Following this idea, Weeks [(*cf.* ARCHER AND AUDLEY (1967) and KEKWICK (1965)] have raised the possibility that latex contains at least two pools of acetyl-CoA not in complete equilibrium. Pyruvate derived acetyl-CoA would be intramitochondrial and preferentially oxidised, while acetyl-

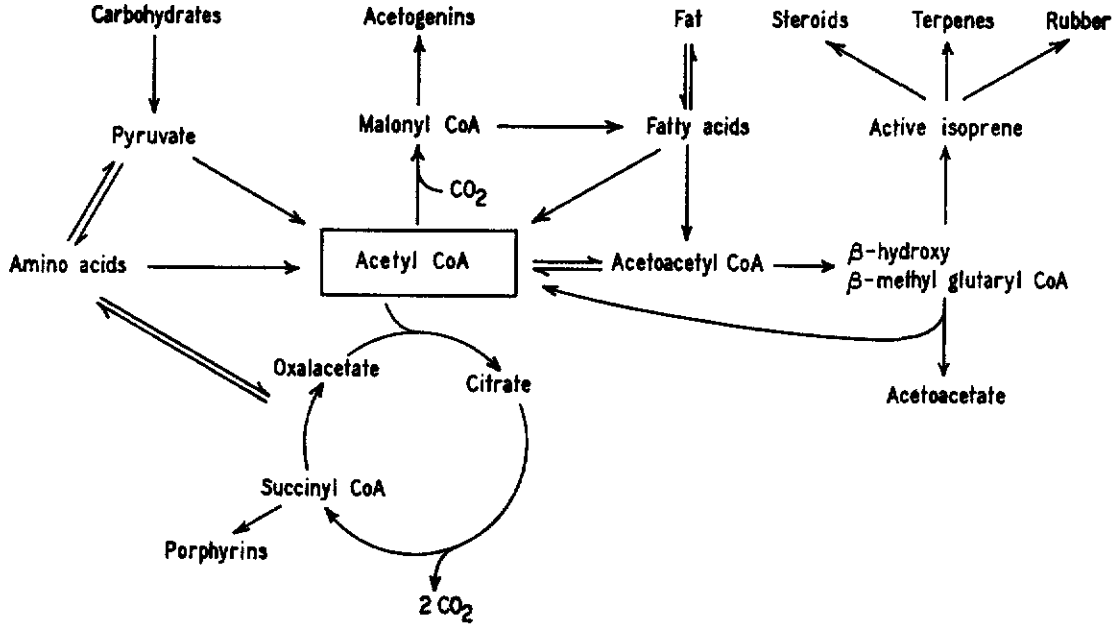


Figure 15. The central position of acetyl-CoA in cellular metabolism.

CoA arising from acetate would be extramitochondrial and used for rubber synthesis.

It is well known that acetyl-CoA occupies a central position in cellular metabolism and that it is generated by a number of catabolic reactions *(Figure 15)*. The oxidation of carbyhydrates and fatty acids and the degradation of amino acids need not stop at the acetyl-CoA level, however, but can proceed to the complete combustion of these molecules yielding CO_2 and H_2O. Any synthetic pathway requiring acetyl-CoA must therefore draw this substance from a pool in competition with other enzymes. The generation and utilisation of acetyl-CoA must balance, and as mentioned above, there may be several physically distinct acetyl-CoA pools, but within these limits, the amount of acetyl-CoA flowing into rubber may be controlled by relative acetyl-CoA binding capacities of the competing enzyme systems. For this reason, the enzymes which prepare acetyl-CoA for incorporation into a specific end-product are very important.

ENZYMIC CATALYSIS — TWO REGULATORY MECHANISMS KNOWN

At this point it may be appropriate to make a few general remarks about the regulation of enzymic catalysis. Two mechanisms have been well documented. A cell can regulate its metabolism by controlling the number of molecules of a particular enzyme in the cell. This regulation may occur at the m-RNA level either by limitations on the rate of transcription or by stimulation of m-RNA degradation, or it may occur at the translation level. The control of enzyme synthesis is referred to as induction or repression (*cf.* AMES AND MARTIN, 1964). Alternatively, the cell can control the catalytic potential of already existing enzymes by end-product inhibition or substrate stimulation. It was found that the enzymes most susceptible to this second type of control stand at the beginning of a specific metabolic sequence. KREBS (1954) called the slowest step in a sequence the reaction catalysed by the 'pacemaker enzyme'. When the end-product exerts an inhibitory effect on the initial enzyme in a pathway lead-

ing specifically to this product, the type of control is referred to as 'feed back inhibition'. In view of the fact that there is often no structural similarity between the inhibitor and the enzyme substrate, it became obvious that modulations of enzyme activity in this case was fundamentally different from classical competitive inhibition and required a specific 'allosteric site' in the words of MONOD *et al.* (1963) for binding of inhibitor. d'Auzac has obtained evidence that allosteric inhibitions and activations play an important role in the control of carbohydrate metabolism of latex. We shall discuss his studies in this symposium. Whether the latex enzymes specifically involved in rubber synthesis from acetyl-CoA are under allosteric regulation is not yet known.

In a preliminary investigation of this question, experiments were designed to measure quantitatively the individual enzymic activities involved in the conversion of acetate to rubber. The experimental techniques used are summarised in the following *Figures*. The formation of acetyl-CoA from acetate and coenzyme A, catalysed by acetyl-CoA synthetase was measured in the usual way (BERG, 1962) by trapping the thioester with hydroxylamine *(Figure 16)*. Thiolase, the enzyme catalysing the reversible cleavage of acetoacetyl-CoA with CoA, thus forming two molecules of acetyl-CoA, was determined in an optical assay

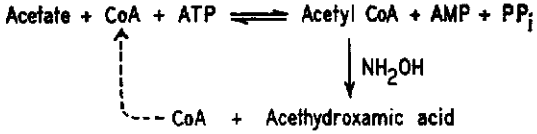


Figure 16. The principle involved in the assay of acetyl-CoA synthetase.

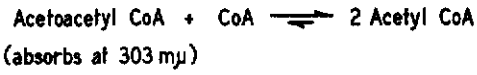


Figure 17. The principle involved in the assay of thiolase.

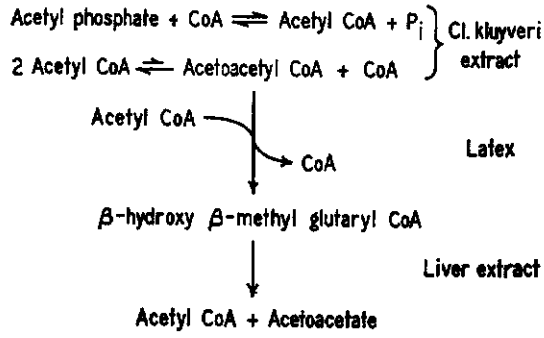


Figure 18. The principle involved in the assay of HMG-CoA synthase.

according to the procedure of STERN (1955) *(Figure 17)*. To measure the activity of the HMG-CoA synthase in latex *(Figure 18)*, an aliquot of the serum fraction is mixed with *Cl. kluyveri* extract (source of excess phosphotransacetylase and thiolase) and heated liver extract (source of HMG-CoA lyase). In this reaction mixture acetyl phosphate is transformed into acetoacetate which can be determined. The acetoacetate formed is proportional to the amount of HMG-CoA synthase present (BUCHER *et al.*, 1960).

The tracer technique was used in order to determine the activities of HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase and pyrophosphomevalonate decarboxylase. This method was first used by TADA AND LYNEN (1961) and is based on the fact, that the carboxyl groups of 5-C^{14}-HMG-CoA, 1-C^{14}-mevalonate or 1-C^{14}-phosphomevalonate are released as $C^{14}O_2$ when the substrates are transformed into isopentenyl pyrophosphate *(Figure 19)*. The reaction was performed in Warburg flasks which contained in the main compartment the radioactive substrate. For the determination of each of these latex enzymes, all the enzymes which are required for the formation of isopentenyl pyrophosphate, except the one to be determined, were added in purified form and in excess. The side arm contained trichloroacetic acid which was tipped into the reaction mixture after incu-

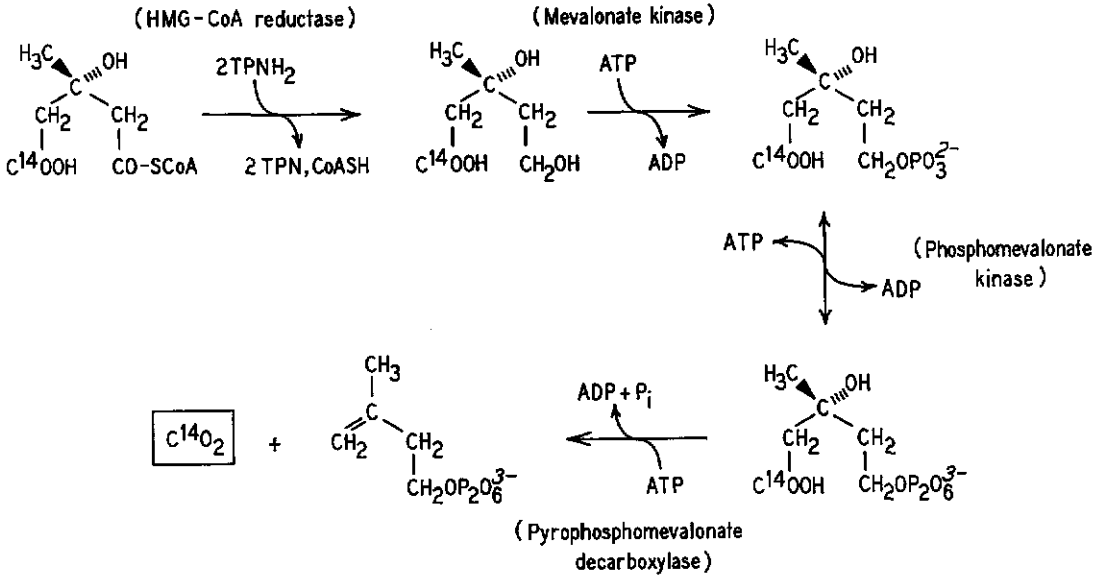


Figure 19. The principle involved in the assay of HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase and pyrophosphomevalonate decarboxylase.

bation. The radioactivity of the released $C^{14}O_2$ was measured after trapping in a solution of hyamnie, present in the centre well of the Warburg flask. Examples of the measurements of HMG-CoA reductase, of phosphomevalonate kinase and pyrophosphomevalonate decarboxylase are shown in *Figures 20* and *21*.

The relative enzymatic activities found by the methods are summarised in *Table 4*. It should be mentioned that all of the enzymes determined, with the exception of polymerase, were present in the serum fraction. As can be seen from *Table 4*, all enzyme activities are of comparable magnitude with the exception of the HMG-CoA reductase. This enzyme activity, which is responsible for the formation of mevalonic acid is much lower, and it is conceivable that the constitutive level of this enzyme may be a limiting factor in rubber biosynthesis. From the comparative point of view it is interesting to note that HMG-CoA reduction is definitely the rate limiting step of cholesterol synthesis in the mammalian organism (BUCHER *et al.*, 1960; SIPERSTEIN AND FAGAN, 1966).

Two other comments should be made. Isopentenyl pyrophosphate isomerase activity has not been measured. The presence of this enzyme in latex is indicated by the fact that freshly tapped latex can convert C^{14}-mevalonic acid not only into rubber, but also into cyclic triterpenes and carotenoids without further additions (*cf.* ARCHER AND AUDLEY, 1967). However, as long as the isomerase activity in latex has not been determined the possibility remains that this enzyme is the 'pacemaker enzyme' of rubber synthesis as suggested by BARNARD (1965). Secondly, thiolase activity as measured by acetoacetyl-CoA formation from acetyl-CoA satisfies the requirements of rubber synthesis. It is present in excess over the HMG-CoA reductase, especially if one considers the relatively high activity of the HMG-CoA synthase, which can trap the acetoacetyl-CoA. The number in bracket *(Table 4)* gives the activity

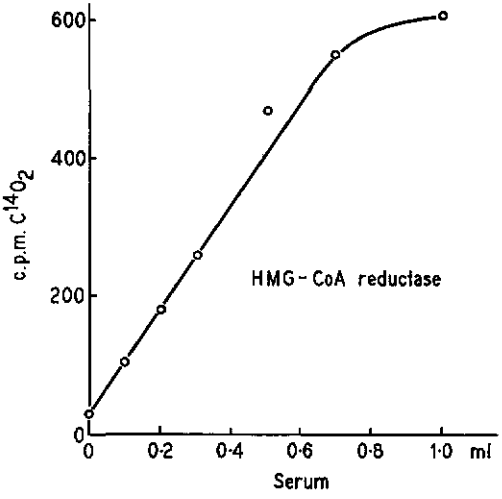


Figure 20. Determination of HMG-CoA reductase in latex serum.

The main compartment of a Warburg flask contained in a total volume of 2.4 ml: 600 µmoles of TRIS-buffer, pH 8.0; 5 µmoles of MgK_2-ethylenediamine tetraacetate; 4 µmoles of $MgCl_2$; 20 µmoles of glutathione; 10 µmoles of ATP; 10 µmoles of glucose-6-phosphate; 2 µmoles of TPN; 50 µg of glucose-6-phosphate dehydrogenase; 75 µg of purified mevalonate kinase; 60 µg of purified phosphomevalonate kinase; 170 µg of purified pyrophosphomevalonate decarboxylase (all enzymes purified from yeast); 0.133 µmole of 5-C^{14}-HMG-CoA (21 700 c.p.m.) and latex serum, as indicated on the abscissa.

After incubation for 3 hours at 37°C, the enzymic reaction was stopped by tipping 0.2 ml of 3 M trichloroacetic acid from the side arm. The $C^{14}O_2$ released was absorbed in 0.15 ml of 1 M hyamine in methanol, soaked in filter paper which was present in the centre well.

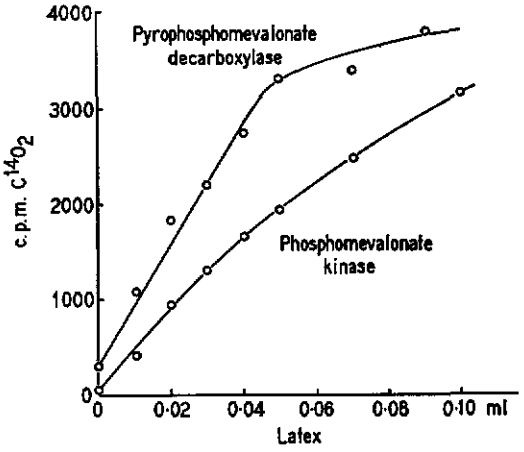


Figure 21. Determination of phosphomevalonate kinase and pyrophosphomevalonate decarboxylase in latex.

The main compartment of a Warburg flask contained in a total volume of 1 ml: 100 µmoles of TRIS buffer, pH 8.0; 6 µmoles of MgK_2-ethylenediamine tetraacetate; 6 µmoles of $MgCl_2$; 2 µmoles of ATP; 0.18 µmole of 1-C^{14}-phosphomevalonate (6000 c.p.m.); an aliquot of latex, as indicated on the abscissa and either 170 µg of purified yeast pyrophosphomevalonate decarboxylase or 60 µg of purified yeast phosphomevalonate kinase (enzyme preparations cf. TADA AND LYNEN, 1961*). After incubation for 45 minutes at 37°C, the enzymic reaction was stopped by the addition of 0.2 ml of 3 M trichloroacetic acid from the side arm. The $C^{14}O_2$ released was absorbed in 0.15 ml of 1 M hyamine solution (in methanol) which was present in the centre well together with filter paper. The radioactivity of the hyamine solution was measured after 2 hours in a Packard liquid scintillation counter.*

of thiolase measured in the opposite direction, that is, in the direction of thiolytic cleavage of acetoacetyl-CoA. This activity was found to be one thousand times higher than the forward reaction. Consequently, the production of acetoacetyl-CoA by thiolase is not the rate limiting step in rubber biosynthesis and the expenditure of ATP to accelerate acetoacetyl-CoA formation *via* malonyl-CoA is not necessary. From these measurements, we come to the conclusion that malonyl-CoA is not an obligatory intermediate of rubber biosynthesis.

TABLE 4. ACTIVITY OF ENZYMES INVOLVED IN RUBBER SYNTHESIS FROM ACETATE

Enzyme	Enzyme activity at 37°C (mμmoles of substrate consumed/min/ml latex)
Acetyl-CoA synthetase	59
Thiolase	3.92 (3920*)
HMG-CoA synthase	232
HMG-CoA reductase	0.078
Mevalonate kinase	149
P-mevalonate kinase	44
PP-mevalonate decarboxylase	103
Isopentenyl-PP isomerase	–
Polymerase	22.3

*Thiolase activity was measured in the reverse direction.

SUMMARY AND CONCLUSIONS

In summary, the pathway of rubber synthesis, as far as intermediates and enzymes are concerned, has been rather well described, but an investigation of the factors controlling rubber production is at its initial stage. The maximum constitutive levels of the various enzymes required for isoprenoid synthesis may well impose serious restriction on the speed with which end-product is formed. Within this framework, however, regulation of specific enzymatic activities by small molecules and end-product undoubtedly plays an important role, especially in the formation and utilisation of acetyl-CoA. Whether any of the enzymes mentioned is inducible has not been determined, but knowledge pertaining to such a regulatory mechanism would find immediate application.

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