Analogues of Geranyl Pyrophosphate as Inhibitors of Prenyltransferase

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Six analogues of geranyl pyrophosphate (the monophosphates of geraniol and tetrahydrogeraniol, and the pyrophosphates of nerol, octan-1-ol, tetrahydrogeraniol and citronellol) were synthesized, and were found to be inhibitors of pig liver prenyl- (geranyl-)transferase. The effects of each analogue were analysed in kinetic experiments, which showed the pyrophosphates of citronellol, tetrahydrogeraniol and octan-1-ol to be the most potent inhibitors. The results are interpreted to support a previous hypothesis that the main forces in the binding of substrates to prenyltransferase are non-specific lipophilic forces and a pyrophosphate-binding force.

Previous experiments with prenyltransferase (EC 2.5.1.1) led to the conclusion that the mechanism of the reaction catalysed by this enzyme was ordered and sequential, described by Scheme 1 [where E is enzyme, S₁ and S₂ are geranyl pyrophosphate and isopentenyl pyrophosphate respectively, P₁ is farnesyl pyrophosphate and P₂ is inorganic pyrophosphate (Holloway & Popják, 1967)]. On the basis of this mechanism, as well as on the previously deduced stereochemistry of the reaction (Cornforth, Cornforth, Donninger & Popják, 1966), it was possible to define the alignment of the substrates on the enzyme and to propose that the substrates were bound in a groove of the enzyme, of which one side contained the binding site for the pyrophosphoryl group of the first substrate (allyl pyrophosphate) and the other side consisted of lipophilic groups holding the alkyl residues of the substrates (Holloway & Popják, 1967).

To define more clearly the properties of prenyltransferase and the requirements for the binding of a molecule to the active sites of this enzyme, we decided to examine the effects of analogues of geranyl pyrophosphate on the kinetics of the

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prenyltransferase reaction. An object of the work was also to achieve a rational approach to designing inhibitors of cholesterol biosynthesis.

MATERIALS AND METHODS

Geranyl pyrophosphate, [4-¹⁴C]- and [1-¹⁴C]-isopentenyl pyrophosphate. These preparations were those described and used previously (Holloway & Popják, 1967; Donninger & Popják, 1967), except that the [1-¹⁴C]isopentenyl pyrophosphate preparation, which was contaminated with the monophosphate, was purified by chromatography on a column (8 cm. × 1 cm.) of DEAE-cellulose (Whatman DE-11 cellulose) equilibrated with aq. 80 mm-ammonium formate, which was also the first eluting solvent. The chromatography of 5.18 mg. of the lithium salt of the impure [1-¹⁴C]-isopentenyl pyrophosphate on this column is shown in Fig. 1. The first radioactive peak, eluted between 12 and 22 ml. of effluent, was isopentenyl monophosphate. The substance eluted with 80 mm-ammonium formate in aq. 0.2 N-NH₃ (see Fig. 1) proved to be isopentenyl pyrophosphate and was found by thin-layer chromatography to be chemically and radiochemically pure, and gave a correct analysis for phosphorus. The appropriate fractions from the column chromatography were pooled and freeze-dried. Since in most experiments very low concentrations (1–8 μM) of isopentenyl pyrophosphate had to be used, the specific radioactivity (0.069 μC/μmole) of the synthetic substrate was raised to 0.43 μC/μmole by mixing it with [4-¹⁴C]-isopentenyl pyrophosphate (specific radioactivity 5.03 μC/μmole) formed enzymically from [2-¹⁴C]mevalonate (Holloway & Popják, 1967); a 10 mm solution in aq. 0.01 N-NH₃ was used in the experiments.

Analogues of geranyl pyrophosphate. The analogues of geranyl pyrophosphate made were the monophosphate of geraniol and the mono- and pyro-phosphate of nerol (*cis*-geraniol), citronellol, tetrahydrogeraniol, 6,7-dihydrogeraniol and octan-1-ol. These were made by phosphorylating the alcohols, on a 0.5–1.0 millimolar scale, by the method

Scheme 1.

Fig. 1. Purification of synthetic $[1-^{14}\text{C}]$ isopentenyl pyrophosphate on a DEAE-cellulose column. The first solvent was aq. 80 mM-ammonium formate; at the arrow this was changed to 80 mM-ammonium formate in aq. 0.1 N-ammonia. IP, isopentenyl phosphate; IPP, isopentenyl pyrophosphate.

of Cramer & Böhm (1959) as modified by Popják Cornforth, Cornforth, Ryhage & Goodman (1962) for the phosphorylation of farnesol.

The procedure introduced by Holloway & Popják (1967) for the isolation and purification of farnesyl mono- and pyro-phosphate, including the use of Amberlite XAD-2 polystyrene resin (Rohm and Haas Co., Philadelphia, Pa., U.S.A.) for the differential absorption of the organophosphorus compounds from an aqueous solution, was applied also to the analogues of geranyl pyrophosphate, except that for the separation of the mono- and pyro-phosphate on the DEAE-cellulose column the aqueous solvents described here for the purification of isopentenyl pyrophosphate were employed.

The purification and fractionation of the analogues of geranyl pyrophosphate by chromatography on DEAE-cellulose were followed (Holloway & Popják, 1967) on Eastman Kodak Co. Chromatoplates (type K301,R2; reclassified as type 6061) with two solvent systems: (A) propan-2-ol-aq. NH_3 (sp.gr. 0.88)-water (6:3:1, by vol.) and (B) ethanol-aq. NH_3 (sp.gr. 0.88)-water (15:4:3, by vol.), the phosphorus-containing spots being rendered visible by spraying the sheets with the acid-molybdate reagent of Hanes & Isherwood (1949) followed by a spray of aq. 5% (w/v) ascorbic acid solution. All parts of the atomizer in contact with these reagents must be either glass or an inert plastic.

With both solvent systems inorganic phosphate remains at the origin. A clear differentiation with either solvent system is possible only between the mono- and pyrophosphoryl derivative of any one alcohol or between the corresponding derivatives of a C_5 and a C_{10} (or a C_8 or a C_{15}) alcohol. The separation factor between the mono- and

pyro-phosphate of a given alcohol is about 1.15, the former migrating further than the latter. R_F values with solvent B for representative samples were: isopentenyl pyrophosphate and 3,3-dimethylallyl pyrophosphate, 0.3-0.36; octyl monophosphate, 0.70; octyl pyrophosphate, 0.61; 7-6, dihydrogeranyl monophosphate, 0.72; 6,7-dihydrogeranyl pyrophosphate, 0.63; geranyl pyrophosphate, 0.68. The derivatives of citronellol and tetrahydrogeraniol behaved like those of 6,7-dihydrogeraniol.

The yields of the mono- and pyro-phosphoryl derivatives of the various alcohols were only about 5% each, lower than those found before in similar syntheses of dimethylallyl pyrophosphate and farnesyl pyrophosphate (see Holloway & Popják, 1967; Popják *et al.* 1962). Since the completion of this work, it was found that whereas the XAD-2 resin absorbed well the phosphorylated derivatives of farnesol, which could be eluted from the resin only with methanolic 0.01 N- NH_3 , the lower homologues, being less lipophilic, were partly eluted from the resin even with aq. 0.01 N- NH_3 ; this accounts partly for the low yields. Nevertheless, the use of the XAD-2 resin has the virtue of removing all inorganic phosphate, which is an inhibitor of prenyl-transferase, at an early stage of purification of the phosphorylated alcohols.

All the specimens of the mono- and pyro-phosphoryl derivatives of the various alcohols used in the experiments were free of inorganic phosphate and contained only one component on analysis by thin-layer chromatography. The preparations were standardized by analysis for phosphorus (Goodman & Popják, 1960).

Although it was known from previous work that the method of Cramer & Böhm (1959) did not cause isomerization during the phosphorylation of geraniol or of *trans-trans*-farnesol, this was checked again by the hydrolysis of the synthetic pyrophosphates of geraniol, nerol, 6,7-dihydrogeraniol and citronellol with intestinal alkaline phosphatase, followed by the analysis of the liberated alcohols by gas-liquid chromatography. The methods used were the same as those applied for the analysis of farnesyl pyrophosphate (Goodman & Popják, 1960; Holloway & Popják, 1967). We obtained no evidence of any isomerization of the alcohols during the phosphorylation.

Sources of prenols and their analogues. Geraniol and nerol, the pure *trans*- and *cis* isomers respectively, were the products of A. Boake, Roberts and Co. Ltd., London, E. 17. Tetrahydrogeraniol (3,7-dimethyloctan-1-ol) was a gift of Bush Boake Allen Ltd., London, N. 1; it contained a single component on gas-liquid-chromatographic analysis and both nuclear-magnetic-resonance spectra and mass spectra fully supported the structure of tetrahydrogeraniol. Pure ($\pm$)-citronellol was obtained by preparative gas-liquid chromatography at 140° from a commercial preparation (British Drug Houses Ltd., Poole, Dorset). The octan-1-ol used was a gas-liquid-chromatographic reference compound supplied by Polyscience Corp., Evanston, Ill., U.S.A. The preparation of 6,7-dihydrogeraniol is described in the preceding paper (Popják, Holloway & Baron, 1969).

All the alcohols used for the preparation of the phosphorylated derivatives could be well characterized by gas-liquid chromatography; their retention volumes relative to that of geraniol (R_g 1.0) on a column of 10% Carbowax 20M (cf. Popják *et al.* 1969) at 135° were: octan-1-ol, 0.32; tetrahydrogeraniol, 0.44; 6,7-dihydronerol, 0.58; 6,7-dihydrogeraniol, 0.66; citronellol, 0.70; nerol 0.81.

Prenyltransferase. The enzyme preparation, made from pig liver (Holloway & Popják, 1967), had initially a specific activity of 75, but in the course of the work it deteriorated to a specific activity of about 60 mμmoles of farnesyl pyrophosphate synthesized/min./mg. of protein (see the experiment with tetrahydrogeranyl phosphate, Table 2). All kinetic experiments were made as described by Holloway & Popják (1967) in incubations of 1 ml., which contained 20 μmoles of tris-HCl buffer, pH 7.9, 5 μmoles of MgCl₂, geranyl pyrophosphate (final concn. 1.1, 1.7, 3.0 or 8.0 μM), a mixture of [¹⁴C]- and [4-¹⁴C]-isopentenyl pyrophosphate (950 disintegrations/min./mμmole; 1.14, 1.9, 3.0 or 5.7 μM) enzyme, and 12.5 μg. of (specific activity 75; in the experi-

ments with tetrahydrogeranyl phosphate the specific activity of the enzyme was about 60). The analogues of geranyl pyrophosphate were added to these standard incubations for testing their effects on the prenyltransferase. The incubations were made at 37° for 2 min., during which time less than 50% of the substrates was utilized and the reaction rate remained essentially linear with respect to time; therefore the amount of product formed during the incubation period was taken as a measure of the initial reaction velocity. The reaction product ([¹⁴C]farnesyl pyrophosphate) was determined, and assays of ¹⁴C by liquid-scintillation counting were carried out, as described by Holloway & Popják (1967).

RESULTS

Table 1. *Effects of analogues of geranyl pyrophosphate on prenyltransferase*

The conditions of the experiments were those described in the Materials and Methods section, except that the concentrations of geranyl pyrophosphate and [¹⁴C]isopentenyl pyrophosphate were 3.00 and 1.14 μM respectively. Analogues of geranyl pyrophosphate were added in the concentrations shown.

Analogue	Concn. of analogue in incubation (μM)	Inhibition of initial reaction rate (%)
Geraniol	100	7
Nerol	100	5
Monophosphates		
Geraniol	18	52
Tetrahydrogeraniol	22	52
Pyrophosphates		
Nerol	10	48
Octan-1-ol	4.4	54
Tetrahydrogeraniol	1.8	43
Citronellol	0.96	49
6,7-Dihydrogeraniol	5.2	29

Effects of analogues of geranyl pyrophosphate on prenyltransferase. The results of some of the earliest orienting experiments with the analogues of geranyl pyrophosphate showed that every one of these inhibited the prenyltransferase at quite low concentrations (Table 1). In contrast with the phosphorylated compounds, the free alcohols, geraniol and nerol, had a barely detectable effect even at a concentration of 100 μM.

A detailed kinetic analysis was made of the effects of the inhibitors, excepting those of 6,7-dihydrogeraniol, because this substance turned out to be a substrate of prenyltransferase. Detailed experiments with 6,7-dihydrogeraniol are reported by Popják *et al.* (1969).

Kinetic experiments with pseudogeranyl pyrophosphates. The effects of each inhibitor on the initial reaction velocity (*v*) of prenyltransferase were determined in two sets of 16 incubations at four concentrations of inhibitor, [*I*]. In one set, in which the initial concentration of isopentenyl pyrophosphate, [*S*₂], was constant at 1.14 μM, the concentrations of geranyl pyrophosphate, [*S*₁], were 1.1, 1.7,

Table 2. *Effects of geranyl monophosphate and tetrahydrogeranyl monophosphate on observed rates of the prenyltransferase reaction*

When geranyl pyrophosphate was the substrate varied, the concentration of isopentenyl pyrophosphate was constant at 1.14 μM, and when the latter was varied, the concentration of geranyl pyrophosphate was 3 μM. The experimental conditions were those described in the Materials and Methods section. *v*, rate of prenyltransferase reaction.

Substrate varied	Concn. of varied substrate (μM)	Inhibitor Concn. of inhibitor (μM) ...	<i>v</i> (mμmoles/min.)							
			Geranyl phosphate				Tetrahydrogeranyl phosphate			
			0	4.5	9.0	18.0	0	4.4	8.8	17.6
Geranyl pyrophosphate	1.1		10.1	7.8	7.7	5.2	8.7	7.5	6.6	4.9
	1.7		15.0	11.9	9.8	7.2	13.6	10.8	8.9	6.8
	3.0		19.4	15.8	13.3	9.6	18.9	14.3	11.5	9.2
	8.0		25.1	20.0	17.9	12.8	23.2	17.2	14.8	11.1
Isopentenyl pyrophosphate	1.14		18.9	15.6	12.2	9.3	14.0	12.7	10.4	7.8
	1.90		22.3	17.7	15.7	11.8	17.8	16.0	13.9	11.0
	3.00		25.0	19.8	17.4	13.3	18.1	17.0	14.9	11.9
	5.70		26.7	22.2	18.8	15.0	21.8	18.6	16.6	14.5

Table 3. Effects of the pyrophosphates of nerol, octanol, tetrahydrogeraniol and citronellol on observed rates of the prenyltransferase reaction

Conditions were as described in Table 2. v , rate of prenyltransferase reaction. v (mμmoles/min.)

Substrate varied	Concn. of varied substrate (μM)	Inhibitor ... Concn. of inhibitor (μM)	Neryl pyrophosphate				Octyl pyrophosphate				Tetrahydrogeranyl pyrophosphate				Citronellyl pyrophosphate			
			0	3.2	6.4	12.8	0	1.1	2.2	4.4	0	0.9	1.8	3.6	0	0.48	0.96	1.92
Geranyl pyrophosphate	1.1	...	9.7	8.4	6.4	4.9	10.5	8.3	6.5	5.1	10.9	8.1	5.9	4.1	10.8	7.5	6.8	4.5
	1.7	...	14.7	11.8	9.0	6.2	15.1	11.6	9.4	7.0	15.7	11.8	8.5	6.1	16.3	11.9	9.4	6.5
	3.0	...	18.9	15.2	12.3	8.4	20.2	15.3	12.5	8.9	21.2	15.1	11.2	7.4	21.5	15.9	13.9	9.2
	8.0	...	24.5	20.3	17.3	12.9	24.9	19.8	17.0	11.9	24.5	19.7	16.0	12.1	25.9	21.9	19.0	15.2
Isopentenyl pyrophosphate	1.14	...	20.3	16.0	12.8	8.4	18.3	14.9	11.7	8.9	19.1	15.9	12.3	8.0	19.8	13.6	12.5	9.1
	1.90	...	24.0	18.9	14.4	10.6	23.0	18.5	14.7	11.6	23.7	18.3	12.8	8.9	24.1	16.9	13.2	10.0
	3.00	...	26.0	20.6	17.0	12.1	26.1	20.2	16.1	13.2	26.2	19.7	14.7	9.7	26.2	18.6	14.7	10.3
	5.70	...	26.9	23.3	18.9	13.6	28.4	21.5	18.6	15.0	29.1	21.1	15.2	10.7	28.8	18.3	14.8	10.2

3.0 and 8.0 μM, the inhibitor concentrations, [I], being 0, 1, 2I and 4I. In the second set of 16 incubations a similar procedure was adopted except that the concentration of S₁ was kept constant at 3 μM and the concentrations of the inhibitor were again 0, 1, 2I and 4I. The values of I for each inhibitor, determined in preliminary experiments as being the most suitable, were as follows: geranyl monophosphate, 4.5 μM; tetrahydrogeranyl monophosphate, 4.4 μM; neryl pyrophosphate, 3.2 μM; octyl pyrophosphate, 1.1 μM; tetrahydrogeranyl pyrophosphate, 0.9 μM; citronellyl pyrophosphate, 0.48 μM. The results are shown in Tables 2 and 3. These were analysed first graphically and then by a computer with the aid of a 'non-linear least-squares' programme (see the Appendix). In the graphical

Fig. 2. Inhibition of prenyltransferase by citronellyl pyrophosphate. The reciprocals of initial rates were plotted against the reciprocal concentrations of the varied substrate: S₁, geranyl pyrophosphate (a); S₂, isopentenyl pyrophosphate (b). Inhibitor concentrations (μM) were: O, 0.0; □, 0.48; △, 0.96; ▽, 1.92.

Fig. 3. Inhibition of prenyltransferase by geranyl monophosphate. The plots are as in Fig. 2. (a) S_1 , geranyl pyrophosphate; (b) S_2 , isopentenyl pyrophosphate. Inhibitor concentrations (μM) were: $\circ$, 0.0; $\square$, 4.5; $\triangle$, 9.0; ∇ , 18.0.

method the reciprocals $1/v$ were plotted against the reciprocals of the concentrations of the substrate varied, $1/[S_1]$ or $1/[S_2]$. This graphical approach revealed that the pseudogeranyl pyrophosphates fell into two groups: (a) the pyrophosphates of tetrahydrogeraniol and citronellol; (b) the pyrophosphates of nerol and octanol, and the monophosphates of geraniol and octanol. Figs. 2 and 3 represent experiments with compounds from group (a) and from group (b) respectively. The obvious difference between the two groups of inhibitors is that the two compounds of group (a) changed primarily the slope of the $1/v = f(1/[S_1])$ lines and affected the intercepts with the ordinate of the $1/v = f(1/[S_2])$ lines only, whereas compounds of group (b) changed both the slopes and the vertical intercepts of the $1/v = f(1/[S])$ lines whether $[S_1]$ or $[S_2]$ was varied.

DISCUSSION

Before the experimental results could be analysed, it was necessary to establish, with reasonable certainty, the correct rate equation and the value of its constants for the uninhibited geranyltransferase reaction represented by the mechanism in Scheme 1. The rate equation for this mechanism, if secondary reactions do not intervene, is equation (1) (Cleland, 1967):

$$v = \frac{V_1[S_1][S_2]}{K_{ia}K_b + K_a[S_2] + K_b[S_1] + [S_1][S_2]} \quad (1)$$

where v is the observed initial reaction velocity, V_1 is the maximum reaction velocity in the forward direction, $[S_1]$ and $[S_2]$ are the concentrations of the substrates binding in succession, geranyl pyrophosphate and isopentenyl pyrophosphate respectively, K_{ia} is the inhibition constant defined by Cleland (1963b) and is equal to k_{-1}/k_{+1} (see Scheme 1), and K_a and K_b are the Michaelis constants for geranyl pyrophosphate and isopentenyl pyrophosphate respectively.

We have re-examined the data in Table 4 of Holloway & Popják (1967), and although our value for K_b , $1.42 \pm 0.34 \mu M$, agrees well with the published figure, we found by both a graphical and a computer analysis (see the Appendix) that their data give in fact a value of $K_a = 3.6 \pm 0.84 \mu M$ and not $4.34 \pm 1.7 \mu M$ as they reported (see also Fig. 4 of the present paper).

Holloway & Popják (1967) did not determine the value of K_{ia} , neither did they examine the possibility that the allylic pyrophosphate substrate might react with a form of the enzyme other than the free enzyme. Since geranyl pyrophosphate is an intermediary product (if dimethylallyl pyrophosphate + isopentenyl pyrophosphate are offered as substrates) as well as a substrate for this enzyme, it was not unreasonable to suppose that it might react not only with the free form of the enzyme but also with the EP_2 complex. If this were the case, assuming the thermodynamic equilibrium:

equation (1) should be modified by multiplying the $[S_1][S_2]$ term in the denominator by $(1 + [S_1]/K_7)$.

For the experimental condition when $[S_1]$ is kept constant and $[S_2]$ is varied the form of equation (1) modified as above, becomes, in the reciprocal form, equation (2):

$$\frac{1}{v} = \frac{1}{V_1} \left(\frac{K_{ia}K_b}{[S_1]} + K_b \right) \frac{1}{[S_2]} + \frac{1}{V_1} \left(1 + \frac{K_a}{[S_1]} + \frac{[S_1]}{K_7} \right) \quad (2)$$

The slope of this equation:

$$\frac{1}{V_1} \left(\frac{K_{ia}K_b}{[S_1]} + K_b \right)$$

is a linear function of $1/[S_1]$. However, examining the data in Table 4 of Holloway & Popják (1967), we found that the plots of $1/v$ against $1/[S_2]$ at four different fixed concentrations of S_1 (geranyl pyrophosphate) gave four parallel lines, i.e. the slopes were independent of the concentrations of S_1 . The examination was done in two ways: (i) by graphical plotting of the data, and the inspection by eye of the graphs, and (ii) by a computer analysis by the method of Cleland (1963*a,b*) (see Holloway & Popják, 1967). The first method gave a mean slope for all four lines of $0.018/1.2 = 0.015$; the second gave the following values ($\pm$ standard errors) with concentrations of $6.5 \mu\text{M}$, $5 \mu\text{M}$, $4 \mu\text{M}$ and $3 \mu\text{M}$ -geranyl pyrophosphate: 0.0146 ± 0.0016 ; 0.0149 ± 0.0011 ; 0.00134 ± 0.00061 ; 0.0152 ± 0.00196 . The computer analysis thus confirmed the judgement of our eyes that the four lines were parallel within experimental error. For the slope of equation (2) to remain constant at increasing concentrations of S_1 is possible only if the value of K_{ta} is zero or very small. Given the value for K_b of $1.42 \mu\text{M}$ we conclude, however, that a value of K_{ta} smaller than about $0.5 \mu\text{M}$ could not have been determined from the

experimental data. By using the computer programme described in the Appendix, we obtained from the previously published data $K_{ta} = 0.03 \pm 0.63 \mu\text{M}$, a value without significance.

To examine the possibility that geranyl pyrophosphate might interact with the EP_2 complex we made further use of the data of Holloway & Popják (1967) and plotted the vertical intercepts of the four parallel lines referred to above either against $[S_1]$ or against $1/[S_1]$. Fig. 4 shows that the plots against $1/[S_1]$ gave a straight line and not a curve as expected if equation (2) applied. The vertical intercept of equation (2):

$$\left(\frac{1}{V_1} + \frac{K_a}{[S_1]V_1} + \frac{[S_1]}{V_1 K_7} \right)$$

can be a linear function of $1/[S_1]$ only if the value of $1/K_7$ is zero. Calculation showed that the value of $1/K_7$ must have been less than 0.02 – 0.03 , as any larger value would have introduced an experimentally detectable curvature into the plots shown in Fig. 4. No significant values for $1/K_7$ could be obtained from the computer analysis of the present results either (see below).

It seems then that equation (1) may be simplified to equation (3), at least as an approximation to assist in a graphical analysis of data:

$$v = \frac{V_1[S_1][S_2]}{K_a[S_2] + K_b[S_1] + [S_1][S_2]} \quad (3)$$

Inhibition of the prenyltransferase by compounds that are pseudogeranyl pyrophosphates could occur by reaction with the free enzyme:

and with the EP_2 complex:

and possibly with both. It can be shown that interaction of an inhibitor with the EP_2 complex effects only the vertical intercepts of the $1/v$ versus $1/[S]$ plots whether the concentration of geranyl pyrophosphate or isopentenyl pyrophosphate is varied. On the other hand, reaction of an inhibitor with the free enzyme alone changes only the slope of the $1/v = f(1/[S])$ lines when $[S_1]$ is varied and affects both the slope and the vertical intercept of the double-reciprocal plots when $[S_2]$ is varied. The latter condition is met, however, only if K_{ta} has a finite value; if K_{ta} is zero, or very small, and particularly when K_b is also small, only the vertical intercepts of the $1/v = f(1/[S_2])$ lines will be changed perceptibly by the inhibitor. This can be appreciated from the form of equation (3) modified to

Fig. 4. Intercepts with the ordinate (Z) of the $1/v = f(1/[S_2])$ functions at four values of $[S_1]$ (3, 4, 5 and $6.5 \mu\text{M}$) plotted against $1/[S_1]$ in the uninhibited prenyltransferase reaction. The calculations are from data of Holloway & Popják (1967). The line represents the equation:

$$Z = 1/V_1 + K_a/V_1[S_1]$$

It extrapolates on the abscissa to $1/[S_1] = -0.28 \mu\text{M}^{-1}$ and gives $K_a = 3.57 \mu\text{M}$ and $V_1 = 99 \text{ m}\mu\text{moles/min}$.

account for the reaction of an inhibitor with E and with EP_2 :

$$\frac{1}{v} = \frac{1}{V_1} \left[\frac{K_a}{[S_1]} \left(1 + \frac{[I]}{K_5} \right) + \frac{K_b}{[S_2]} + \left(1 + \frac{[I]}{K_8} \right) \right] \quad (4)$$

For the condition when $[S_2]$ is varied, equation (4) can be rewritten as equation (5), in which only the vertical intercept is affected by $[I]$:

$$\frac{1}{v} = \frac{K_b}{V_1[S_2]} + \frac{1}{V_1} \left(1 + \frac{[I]}{K_5} + \frac{K_a}{[S_1]} + \frac{K_a[I]}{[S_1]K_5} \right) \quad (5)$$

These reactions, coupled with a negligibly small K_{ia} , are the most probable explanations of the experimental observations with citronellyl pyro-

published work) are artificial substrates for prenyltransferase. A further possibility to be considered was that the pseudogeranyl pyrophosphates reacted also with the ES_1 complex, displacing S_2 from the reaction:

The various possible reactions of substrates and inhibitors with the enzyme and enzyme complexes, as discussed, can be summarized by the model shown in Scheme 3.

Assuming that all the reactions in Scheme 3 are thermodynamic equilibria, the model is represented by the rate equation (6):

$$v = \frac{V_1[S_1][S_2]}{(K_{ia}K_b + K_a[S_2]) \left(1 + \frac{[I]}{K_5} + \frac{[I][S_2]}{K_5K_6} \right) + K_b[S_1] \left(1 + \frac{[I]}{K_8} \right) + [S_1][S_2] \left(1 + \frac{S_1}{K_7} \right) \left(1 + \frac{[I]}{K_8} \right)} \quad (6)$$

phosphate and tetrahydrogeranyl pyrophosphate (cf. Figs. 2a and 2b).

The kinetic effects of the pseudogeranyl pyrophosphates may be further complicated by the reaction of the EI complex with S_2 in Scheme 2.

The previous kinetic study of prenyltransferase led to the conclusion that isopentenyl pyrophosphate can bind to the enzyme only after the formation of the ES_1 complex (Holloway & Popják, 1967). It was conceivable that the EI complex formed with the pseudogeranyl pyrophosphates might fulfil the same role as the ES_1 complex in the normal sequence of reactions, except that in the absence of the allylic double bond in these inhibitors, or because of incorrect stereochemistry as in neryl pyrophosphate, a product could not be formed. This argument is supported by the discovery that some analogues of geranyl pyrophosphate, such as the pyrophosphates of 6,7-dihydrogeraniol (Popják *et al.* 1969) and of *trans*- and *cis*-3-ethyl-3-methylallyl alcohol (G. Popják, J. L. Rabinowitz & J. M. Baron, un-

From the analysis of the data of Holloway & Popják (1967) we have already concluded that the value of $1/K_7$ in equation (6) is experimentally indistinguishable from zero, i.e. that the reaction:

did not occur or was observable only at very high concentrations of S_1 . Scheme 2 with the inhibitors could also be excluded because if this operated the plots of the slopes of the $1/v = f(1/[S_2])$ lines against inhibitor concentrations could not have given straight lines, whereas in fact they did.

Analysis of the results of Tables 2 and 3 in terms of equation (6) by the 'non-linear least-squares' computer programme (see the Appendix) did not produce values significantly different from zero for $1/K_7$ or for $1/K_6$. Thus we were forced to consider finally only three reactions with the pseudogeranyl pyrophosphates:

Scheme 3.

After these eliminations, equation (6) can be rewritten, in the reciprocal form, for the experimental conditions when $[S_1]$ or $[S_2]$ was varied, as equations (7) and (8) respectively; these were the only ones to which the experimental data could be fitted satisfactorily. They have, fortunately, the virtue that the values of K_5 and K_9 can be derived with their aid independently of the values of K_a , K_b or V_1 ; a knowledge of the values of the Michaelis constants (and of either K_5 or K_9) is needed for the determination of the values of K_8 .

$$\frac{1}{v} = \frac{K_a}{V_1} \left(1 + \frac{[I]}{K_5}\right) \frac{1}{[S_1]} + \frac{1}{V_1} \left\{ \frac{K_b}{[S_2]} \left(1 + \frac{[I]}{K_9}\right) + \left(1 + \frac{[I]}{K_8}\right) \right\} \quad (7)$$

$$\frac{1}{v} = \frac{K_b}{V_1} \left(1 + \frac{[I]}{K_9}\right) \frac{1}{[S_2]} + \frac{1}{V_1} \left\{ \frac{K_a}{[S_1]} \left(1 + \frac{[I]}{K_5}\right) + \left(1 + \frac{[I]}{K_8}\right) \right\} \quad (8)$$

The slopes of equations (7) and (8), $Y_{(7)}$ and $Y_{(8)}$ respectively, as well as their intercepts, $Z_{(7)}$ and $Z_{(8)}$, are linear functions of $[I]$ (equations 9, 10, 11 and 12).

$$Y_{(7)} = \frac{K_a}{V_1} + \frac{K_a[I]}{V_1 K_5} \quad (9)$$

$$Y_{(8)} = \frac{K_b}{V_1} + \frac{K_b[I]}{V_1 K_9} \quad (10)$$

$$Z_{(7)} = \frac{1}{V_1} \left(\frac{K_b}{K_9[S_2]} + \frac{1}{K_8} \right) [I] + \frac{1}{V_1} \left(1 + \frac{K_b}{[S_2]} \right) \quad (11)$$

$$Z_{(8)} = \frac{1}{V_1} \left(\frac{K_a}{K_5[S_1]} + \frac{1}{K_8} \right) [I] + \frac{1}{V_1} \left(1 + \frac{K_a}{[S_1]} \right) \quad (12)$$

The intercepts of the lines of $Y_{(7)}=f(I)$ and $Y_{(8)}=f(I)$ with the abscissa, i.e. when $Y_{(7)}=0$ and $Y_{(8)}=0$, give the values of K_5 and K_9 . With a knowledge of these two inhibition constants K_8 may be calculated from equations (11) and (12) provided that the values of K_b and K_a are also known. It can be readily deduced that the values of $[I]$ when $Z_{(7)}$ or $Z_{(8)}$ are zero, $[I_{(7)}^0]$ and $[I_{(8)}^0]$ respectively, are related to K_5 , K_8 and K_9 as shown in equations (13) and (14), where $\sigma_1 = K_a/[S_1]$ and $\sigma_2 = K_b/[S_2]$ at fixed values of $[S_1]$ and $[S_2]$ respectively:

$$\frac{\sigma_2}{K_9} + \frac{1}{K_8} = -\frac{1 + \sigma_2}{[I_{(7)}^0]} \quad (13)$$

$$\frac{\sigma_1}{K_5} + \frac{1}{K_8} = -\frac{1 + \sigma_1}{[I_{(8)}^0]} \quad (14)$$

Examples of the graphical analysis of the data by the use of equations (7)–(14) are shown in Figs. 5 and 6, and the values of the inhibition constants are derived in Table 4.

Table 4. *Inhibition constants of analogues of geranyl pyrophosphate for prenyltransferase deduced by graphical analysis of kinetic data*

$$K_5 = \frac{[E][I]}{[EI]}; \quad K_9 = \frac{[ES_1][I]}{[ES_1I]}; \quad K_8 = \frac{[EP_2][I]}{[EP_2I]}$$

It was assumed in the analyses that the value of K_{ia} was less than $0.5 \mu\text{M}$ and therefore negligible, and that $K_a = 3.6 \mu\text{M}$ and $K_b = 1.42 \mu\text{M}$. The method of calculation is illustrated in Figs. 5 and 6.

Inhibitor	K_5 (μM)	K_9 (μM)	K_8 (μM)
Geranyl phosphate	16.0	10.6	> 100
Tetrahydrogeranyl phosphate	13.5	11.0	∞
Neryl pyrophosphate	6.5	5.0	∞
Octyl pyrophosphate	3.35	2.3	∞
Tetrahydrogeranyl pyrophosphate	1.25	∞	1.87
Citronellyl pyrophosphate	0.9	∞	1.16

Fig. 5. Plots of the slopes (a), and of the intercepts (b), of the $1/v=f(1/[S_1])$ lines, at four concentrations of inhibitor, against the concentrations of inhibitor, geranyl phosphate. From graph (a), $K_5 = 16.0 \mu\text{M}$, and from graph (b), $1/K_8 = (2.245/18.4) - (1.245/K_9)$. Since, from Fig. 6(a), $K_9 = 10.6 \mu\text{M}$, $1/K_8 = 0.0045 \mu\text{M}^{-1}$.

Fig. 6. Plots of the slopes (a), and of the intercepts (b), of the $1/v = f(1/[S_2])$ lines, at four concentrations of inhibitor, against the concentrations of inhibitor, geranyl phosphate. From graph (a) $K_9 = 10.6 \mu\text{M}$, and from graph (b) $1/K_8 = (2.2/23.4) - (1.2/K_5)$. Since, from Fig. 5(a), $K_5 = 16.0 \mu\text{M}$, $1/K_8 = 0.094 - 0.075 = 0.009 \mu\text{M}^{-1}$.

The data were also examined by a 'non-linear least-squares' computer programme. Attempts were made to fit the data to equation (6) and its variants, the only restrictions imposed being the values of the Michaelis constants, K_a $3.6 \mu\text{M}$ and K_b $1.42 \mu\text{M}$. No values for $1/K_8$ or $1/K_7$ differing significantly from zero were found in any of the computations, confirming the conclusion of the graphical analyses. Thus it must be concluded that neither Scheme 2 nor the interaction of S_1 with the EP_2 complex occurred, however plausible these seemed. The values of K_5 , K_8 and K_9 calculated by the computer (Table 5) are in reasonably good agreement with those deduced by graphical means. The standard deviations (σ) of the velocity observations about the model indicated mostly good fits. The largest error met was with the data from the experiment with tetrahydrogeranyl pyrophosphate; this was accounted for by experimental errors apparent also in the course of graphical analysis.

The evaluation of the set of data with tetrahydrogeranyl pyrophosphate was difficult both by the graphical method and by the computer. On

plotting $1/v$ against $1/[S_2]$ at various values of $[I]$, the lines obtained at the two lowest concentrations of inhibitor were parallel with the line found in the absence of the inhibitor, but at the highest concentration of tetrahydrogeranyl pyrophosphate the slope increased significantly. In the graphical analysis the value of $1/K_9$ was assumed to be zero. The computer had difficulty in assigning values to $1/K_8$ and $1/K_9$ [see Table 5, line (a) under tetrahydrogeranyl pyrophosphate]: the values computed for these terms had in fact such large standard errors as to render them not significant. In order to see whether either of the two constants was more relevant than the other to the mode of action of tetrahydrogeranyl pyrophosphate, the programme was repeated on this set of data, fixing alternatively $1/K_8$ or $1/K_9$ at zero. The values so derived are shown in lines (b) and (c) in Table 5. Even this calculation showed no real advantage to either term.

We interpret the results of the analyses to mean that citronellyl pyrophosphate, the closest analogue of geranyl pyrophosphate, reacted preferentially with E and with the EP_2 complex ($1/K_9 = 0$), and that tetrahydrogeranyl pyrophosphate reacted probably with three forms of the enzyme: E, ES_1 and EP_2 .

The remaining four analogues appear to have reacted with E and ES_1 , but not at all with EP_2 ($1/K_8 = 0$); i.e. they behaved as analogues of both geranyl pyrophosphate and isopentenyl pyrophosphate. This was unexpected, though not unreasonable in the light of the former suggestion that isopentenyl pyrophosphate was bound to the enzyme secondarily by a phosphate-phosphate interaction, promoted by chelation with Mg^{2+} , between the ES_1 complex and one of the phosphate groups of isopentenyl pyrophosphate. Even with this unexpected conclusion, the results support the previous view (Holloway & Popják, 1967) that the binding of substrates to prenyltransferase is determined mainly by the reactions of the pyrophosphate groups and secondarily by lipophilic forces. The importance of the pyrophosphate group is emphasized by the tenfold difference between the inhibitory powers of the mono- and pyro-phosphate of tetrahydrogeraniol. This is in accord with the observation made during the product-inhibition studies (Holloway & Popják, 1967) where farnesyl pyrophosphate appeared to interact specifically with EP_2 , but inorganic pyrophosphate at high concentrations reacted with more than one enzyme form.

The nature of the alkyl residue seems to be of less consequence, excepting that the ability to take up a conformation similar to that of geraniol favours reactions with the enzyme, as evidenced by the difference between the inhibitory concentrations of

Table 5. *Values of kinetic constants (with their standard errors) of prenyltransferase reaction in the presence of inhibitors calculated by computer from the model*

The model used was:

$$v = V_1[S_1][S_2] / \left\{ (K_{ia}K_b + K_a[S_2]) \left(1 + \frac{[I]}{K_5} \right) + K_b[S_1] \left(1 + \frac{[I]}{K_5} \right) + [S_1][S_2] \left(1 + \frac{[I]}{K_8} \right) \right\}$$

Inhibitor	$V_1 \dagger$ (mμmoles/ min.)	K_{ia} (μM)	$1/K_5$ (μM ⁻¹)	$1/K_8$ (μM ⁻¹)	$1/K_9$ (μM ⁻¹)	Standard deviation about model (σ)
Geranyl phosphate	67.7* (3.7)	0.38 (0.20)	0.072* (0.012)	0.0139 (0.020)	0.063* (0.012)	0.525
Tetrahydrogeranyl phosphate	53.8* (2.3)	-0.21 (0.45)	0.071* (0.034)	-0.022 (0.052)	0.085* (0.030)	1.158
Neryl pyrophosphate	70.3* (1.6)	0.49 (0.27)	0.140* (0.026)	-0.006 (0.044)	0.112* (0.026)	0.711
Octyl pyrophosphate	70.5* (1.3)	0.59* (0.22)	0.31* (0.06)	0.092 (0.095)	0.33* (0.06)	0.564
Tetrahydrogeranyl pyrophosphate‡ (a)	71.7* (1.7)	0.37 (0.27)	0.60* (0.11)	0.34 (0.20)	0.21 (0.12)	0.732
	(b) 73.4* (1.6)	0.68* (0.23)	0.50* (0.09)	0.63* (0.11)	0 (0.06)	0.763
	(c) 69.8* (1.3)	0.55* (0.18)	0.79* (0.05)	0 (0.06)	0.39* (0.06)	0.757
Citronellyl pyrophosphate	71.3* (1.7)	0.29 (0.27)	1.33* (0.20)	0.95* (0.38)	-0.46 (0.25)	0.737

* Significantly different from zero at least at the 5% level.

† The values of V_1 , except in the experiment with tetrahydrogeranyl phosphate, did not differ significantly from one another; their mean value was 70.3 ± 0.97 mμmoles/min.

‡ In computation (b) $1/K_9$, and in computation (c) $1/K_8$, was fixed at zero.

the pyrophosphates of nerol and octanol. The two most potent inhibitors, the pyrophosphates of citronellol and of tetrahydrogeraniol, can attain a conformation closest to that of the natural substrate.

Holloway & Popják (1967) commented on the difficulties inherent in the experimental technique of determining initial reaction rates with prenyltransferase. These difficulties are still with us, and until a more accurate method can be devised the true value of K_{ia} will remain uncertain, for it cannot be credited that the value of this constant is truly zero. The computer analysis gave K_{ia} values generally greater than zero, though in only one set was the value significant (see Table 5). None of the K_{ia} values differed significantly from each other and the mean of the six determinations gave a value of $0.38 \mu\text{M}$, which, with a standard error of ± 0.14 , was significantly greater than zero. The computer thus seems to have vindicated the conclusion drawn from the graphical analysis that the value of K_{ia} must be less than $0.5 \mu\text{M}$. We realize now also that, in order to obtain more reliable kinetic results than those collected on this enzyme to date, a much wider range of substrate concentrations will have to be employed than was possible hitherto.

It was pointed out by Holloway & Popják (1967) that the pattern of product inhibition observed by them with prenyltransferase was compatible not only with the mechanism of Scheme 1 but also with a mechanism termed Iso Ping Pong Bi Bi by Cleland (1963b). The pattern of inhibition seen with the pseudogeranyl pyrophosphates could be explained also by that mechanism, which required, however, that isopentenyl pyrophosphate combined first with the enzyme and that the inorganic pyrophosphate release in the transferase reaction came from isopentenyl pyrophosphate and not from geranyl pyrophosphate. Although this is not entirely impossible, it seems most unlikely on chemical grounds. Admittedly there is no proof available to show, for instance, that the pyrophosphate group in farnesyl pyrophosphate comes from isopentenyl pyrophosphate, although it should not be too difficult to obtain evidence on this question.

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APPENDIX

The statistical evaluation of the data was done by the method of least squares, which finds those values for the constants that minimize the sum of squares of the deviations of the observed velocities from the velocities predicted by the model of the rate equation. The calculations were done on the Univac 1108 computer of the Shell Centre, London, S.E. 1, by using a programme written in Fortran IV by Mr A. Jones of the Thornton Research Centre, 'Shell' Research Ltd., Ellesmere Port, Cheshire. The programme, code-named SPIRAL, is designed to fit non-linear models by a number of iterative techniques. By this programme the two sets of data, obtained with each inhibitor from 16 incubations

each when $[S_1]$ and $[S_2]$ were varied in turn, could be analysed together.

Because of limitations imposed by the experimental design, it was not possible to estimate all the constants separately. After preliminary calculations, including some on independent data for the uninhibited prenyltransferase reaction, it was decided to fix $K_a = 3.6 \mu\text{M}$, $K_b = 1.42 \mu\text{M}$, $K_6 = K_7 = \infty$ and to obtain values for V_1 , K_{ta} , $1/K_5$, $1/K_8$ and $1/K_9$. The reciprocals rather than the values for the last three constants were calculated because the reciprocals give better representations of reaction strength, having zero value for no reaction and high values for favoured reactions.